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EPITHELIAL CELL BEHAVIOUR

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To the man who showed me the stars and helped me reach them,

the wind beneath my wings... my soul mate, my best friend,

my husband.

To the two who taught me unconditional love

and nurture me with everlasting love,

mamma e papa'

To the one who believes in me and embodies my inspiration,

nonna.

To the ones that share my joys and my memories,

Sara, Omar and Dania.

With love and fondness...

Nadia

ABSTRACT

Epithelial cells interact with their surrounding extracellular matrix via integrin receptors. Signals generated in this way control epithelial cell migration, proliferation and apoptosis. Changes in the extracellular matrix, integrin expression and protease secretion occur in wound healing and cancer and thus these systems are thought to play a crucial role in such processes. We hypothesise that the interactions between these key players affect epithelial cell behaviour. We have utilised a variety of epithelial cells including normal primary cells and lines with manipulated integrin expression to investigate this further.

Epithelial cells are commonly grown in medium containing additives such as hydrocortisone, EGF, insulin and cholera toxin. When epithelial cell are cultured in medium without additives they adopt a more mesenchymal phenotype. This was shown to be due to the absence of EGF which resulted in a down-regulation of the cell-cell adhesion molecule e-cadherin.

Experiments were performed examining the interaction of epithelial cells with a variety of fibronectin fragments. The adhesion of normal keratinocytes and cell lines expressing low (C1) and high (VB6) levels of the β6 integrin subunit was unaffected on the 120kDa fibronectin fragment when compared to the full length molecule. Motility has been followed using wound assays and

Transwell® migration assays. The latter showed that migration was significantly increased on the 120kDa fragment but only in the VB6 cells. This may be achieved through the up-regulation of matrix metalloproteinase 9 (MMP-9) which is increased in the supernatant from VB6 cells plated on 120kDa fragment. *In vitro* this fragment can be generated by incubating full length fibronectin with purified MMP-9.

Immunohistochemical studies using oral squamous cell carcinoma tissue indicate that these interactions may occur *in vivo* with fibronectin, β6 integrin and MMPs all being located at the invading tumour front.

We have thus identified an important feedback loop where expression of the β6 integrin subunit which is only seen in wound healing and cancer enhances the motility of and MMP secretion by epithelial cells in contact with the 120kDa fibronectin fragment. These enzymes can then further degrade fibronectin to generate additional fragments. If it were possible to distinguish this fragment from the parent molecule immunologically it could prove to be a valuable marker of aggressive OSCC being indicative of a number of pro-tumourigenic processes.

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Read in the name of thy Lord who created
Created man from a clotted tissue
Read and thy Lord is most Beneficent
Who has taught you by the Pen
Taught man what he knows not

(96: 1-5)

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ABBREVIATIONS

AFM	Additive Free Medium
APES	3-aminopropyltriethoxysilane
BM	Basement membrane
BSA	Bovine Serum Albumin
cFN	Cellular Fibronectin
CS	(variable) connecting segment
CT	Connective Tissue
DAPI	4',6'-diamidino-2-phenyindole, dilactate
DNA	Deoxyribonucleic acid
ECM	Extra-cellular matrix
EDA/B	Extra-domain A/B
EGF	Epidermal Growth Factor
EGFR	Epdermal Growth Factor Receptor
ELC	Enhanced Chemiluminescence
ERK	Extracellular signal regulated kinase
FACS	Fluorescent Activated Cell Sorter
FAK	Focal Adhesion Kinase
FCS	Foetal Calf Serum
FN	Fibronectin
GPI	Glycosylphosphatidylinositol
HBD	Heparin-Binding Domain
HGF	Hepatocyte Growth Factor
IMS	Industrial Methylated Spirit
kDa	Kilo Dalton
KGM	Keratinocyte Growth Medium
LAMMs	Large Apparent Molecular Mass
LAP	Latency Associated Peptide
MAPK	Mitogen Activated Protein Kinase
MMPs	Matrix Metalloproteinases
mRNA	Messenger Ribonucleic Acid
MSF	Macrophage Stimulating Factor

MSF	Migration Stimulating Factor
MT-MMPs	Membrane-type matrix metalloproteinases
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2(4-sulfophenil)-H-tetrazolium
NC	Non-collagenous
NHK	Normal Human Keratinocytes
OA	Osteoarthritis
OSCC	Oral Squamous Cell Carcinoma
PAI	Plasminogen Activator Inhibitor
PBS	Phosphate Buffered Saline
PDGF	Platelet Derived Growth Factor
pFN	Plasma Fibronectin
PG	Prostaglandins
PHRSN	Pro-His-Ser-Arg-Asn
PKC	Protein Kinase C
PMT	Photomultiplier tube
PTK	Protein Tyrosine Kinase
RA	Rheumatoid Arthritis
RGD	Arg-Gly-Asp
SAPK	Stress activation protein kinase
SDS-PAGE	Sodium Dopecyl Sulphate polyacrilamide gel electrophoresis
SFM	Serum Free Medium
SOS	Sons of Sevenless
TBS	Tris Buffered Saline
TGF	Transforming Growth Factor
TIMPs	Tissue Inhibitors of Matrix metalloproteinases
tPA	Tissue Plasminogen Activator
uPA	Urokinase Plasminogen Activator
uPAR	Urokinase Plasminogen Activator Receptor
V/CS	Variable sequence of FN

CHAPTER 1

INTRODUCTION

1.1 STATEMENT OF THE PROBLEM

The environment that surrounds cells is altered in wound healing and carcinogenesis resulting in signals and cues that influence cellular behaviour such as gene expression and motility. These alterations may include the expression of new extracellular matrix (ECM) proteins and the modification of those already present. This can occur by the action of proteolytic enzymes to produce bioactive fragments. In this way molecules that in other circumstances are inert and innocuous may present themselves as inducing a pathological phenotype.

Previous studies have been carried out on fragments of ECM molecules, but few if any have devoted their attention to fragments of fibronectin. Given the abundance of fibronectin in the ECM it is plausible that it may have an important role. Bioactive fragments of fibronectin may be crucial in the process of carcinogenesis by inducing expression of other molecules such as proteases and cell adhesion molecules. Detailed examination of the interplay between ECM fragments, their receptors (integrins) and matrix modifying proteases may identify common pathways which could provide novel ideas

for therapy or may help identify markers of aggressive disease. The challenge is to identify these key pathways in complex interactions that occur temporally and spatially between numerous molecules and cells *in vivo*.

1.2 OVERVIEW

1.2.1 Oral Epithelium

The oral epithelium is the tissue that covers the oral mucosa, underneath which is connective tissue (CT). Like other epithelia in the body it is made up of a large number of cells, the keratinocytes, closely attached to one another to form a continuous barrier. The primary function of oral keratinocytes reflects the function of the oral epithelium, that is, to provide a barrier between the outside environment and the deeper tissues. They therefore serve as protective and selective barriers through which certain substances may pass.

1.2.2 Oral Cancer

Oral squamous cell carcinoma (OSCC) is the commonest of all head and neck tumours accounting for over 90% of mouth malignancies, and is the sixth most common human malignancy (De Vicente 2002). The 5-year survival rate for oral cancer is less than 50% and has not improved in more than two decades (Lippman & Hong 2001). Carcinogenesis is a multistep process. There are three main sequential steps: initiation, promotion and progression.

Clinically, normal tissue first becomes hyperplastic, then dysplastic, followed by the formation of a carcinoma in situ, defined as a lesion that does not extend beyond the basement membrane; subsequently it develops into an invasive carcinoma and finally metastasizes to distant sites by way of blood or lymph. At a molecular level, normal cells first become precancerous, then undergo irreversible DNA changes (immortalization of cancer cells), and finally penetrate the basement membrane and spread. Tumour invasiveness is a distinct characteristic of tumour progression. Once the cells spread beyond the basement membrane, they form an invasive front that interacts with the connective tissue beneath. Tumour cells characteristically exhibit altered cell-to-ECM interactions. They remodel the ECM to facilitate cell migration by increased and modified production of proteases or inhibition of control mechanisms. Of vital importance to this model is the participation of a family of cell-adhesion molecules known as integrins, which link the cells to the molecules in the ECM and provide communication channels. Changes in their profile, localization and signalling is a trait of cancer progression (Kassis *et al.* 2001).

1.3 THE EXTRACELLULAR MATRIX

The development and function of oral keratinocytes, like all other cells in the body, depends on the interactions with molecules in their environment. The

ECM not only provides mechanical support but also influences cell behaviour. Molecules that regulate cellular development and function include growth and differentiation factors, cell adhesion molecules and components of the ECM (Kreis & Vale 1993). During remodelling, whether physiologically or pathologically, new signals are generated between the cells and their environment, particularly the basement membranes (BM) (Ortega & Werb 2002).

1.3.1. The Basement Membrane

Oral keratinocytes rest on a thin mat of specialized ECM known as basement membrane (BM) that separates them from the underlying connective tissue (Raitz *et al.* 2003). The BM varies in composition in a tissue-specific way as well as during repair and development. The BM serves as a structural scaffold and certain components have been identified as regulators of biological activities such as cell growth, migration and differentiation (Erickson & Couchman 2000). Furthermore, it is able to determine cell polarity and influence cell metabolism (Erickson & Couchman 2000). Under the electron microscope it consists of two distinct layers; the *lamina lucida* directly beneath the keratinocytes, and the *lamina densa* just below this. In some cases a third layer may be found consisting primarily of collagen fibrils adjacent to the CT, and this is known as *lamina fibroreticularis*. The composition of the BM varies from tissue to tissue but the constant components are laminins, entactin-1/nidogen-1, type IV collagen and heparin sulphate proteoglycans (Erickson

& Couchman 2000). Anchored to the underlying CT are type VII collagen fibrils (Alberts *et al.* 1994).

During tumourigenesis the first barrier to be broken through is the BM. A primary neoplastic mass can not invade and metastasize to distant loci without first penetrating the supporting BM. By compromising the division between epithelium and connective tissue, the first sign of aggressiveness is established.

1.3.2 Extracellular Matrix Proteins

The extracellular matrix is composed of a large number of molecules. The major components include collagens, fibronectin, vitronectin and laminins. The signalling properties of the ECM depend on the organization of its constituent molecules (Lukashev & Werb 1998). The ECM is increasingly implicated in malignancy via two mechanisms: enzymatic degradation, and the failure of cells to synthesize and assemble the main ECM components (Raitz *et al.* 2003).

1.3.3 Extracellular Matrix Fragments

All the constituent proteins making up the extracellular matrix can be degraded to smaller fragments. This may be as part of a turnover process or may be a specific mechanism whereby smaller bioactive fragments are

generated that carry out distinct and precise functions dissimilar from the parent molecule (Yamada 2000).

1.3.3.1 Collagens and their fragments

Collagens are a large family of glycoproteins. Many forms are found throughout the tissues. Collagens type I and III are the major interstitial, fibrillar collagens that are found in most connective tissues. Collagen types IV and VII are located in the BM. Extensive studies have been carried out on collagen fragments and their implication in both normal physiological processes such as embryogenesis and wound healing, and pathological processes such as carcinogenesis (O'Toole 2001; Ortega & Werb 2002) (Table 1.1).

Collagen type I, II and III, are triple helices made up of polypeptide chains of about 95 kDa, which are quite resistant to degradation by most proteolytic enzymes except collagenases. These proteases belong to the matrix metalloproteinases (MMP) family and are able to cleave all three chains into fragments approximately 75% and 25% of the total length, at specific sites between amino acid residues 775 and 776 (Stringa *et al.* 2000). All of these effects are thought to be mediated via integrins (Pilcher *et al.* 1997; Messent *et al.* 1998; Stringa *et al.* 2000).

Collagen IV, found in all basement membranes, regulates cell adhesion and migration. The non-collagenous C-terminal fragment (NC1) of type IV collagen has been identified as a circulating fragment in serum. This fragment (also a by-product of collagen XV and XVIII degradation) is involved in the regulation of angiogenesis and other morphogenetic processes (Ortega & Werb 2002; Hamano *et al.* 2003).

COLLAGENS	FRAGMENTS	BIOLOGICAL FUNCTIONS
Collagen I	•Trimer Carboxyl Propeptide	Chemotactic for endothelial cells
	•Various fragments	Chemotactic for dermal fibroblasts and peripheral monocytes
Collagen II	**•Various fragments**	**Inhibit collagen synthesis and MMP secretion by chondrocytes.**
	•C-propeptide or Chondrocalcin	**Cartilage calcification**
Collagen IV	•NC1 and 7S domains	Inhibit morphogenesis
	•NC1 domain	Promote axonal growth
	•α1 NC1, arresten	Anti-angiogenic
	•α2 NC1, canstatin	Anti-angiogenic
	•α3 NC1, tumstatin	Anti-angiogenic
	•α6 NC1	Anti-angiogenic
Collagen VIII	**•NC1 domain vastatin**	**Angiogenic**
Collagen XIV	•N-terminal fragment	Chemotactic for neutrophils
Collagen XV	**•NC1**	**Anti-angiogenic**
	•Restin	**Anti-angiogenic**
Collagen XVIII	•NC1	Induce migration of neural and non-neural cells.
	•Endostatin	Anti-angiogenic, inhibit branching Morphogenesis

Table 1.1 Biological functions of various collagen fragments (Ortega & Werb 2002).

1.3.3.2 Laminins and their fragments

Laminins constitute a family of ECM molecules that are associated with BM. They have been found to affect adhesion, migration, growth and the differentiation of cells (Kreis & Vale 1993). Proteolytic degradation of Laminin-5 generates fragments of 160 kDa and 105 kDa that can influence cell adhesion and migration. Such fragments have been detected in human fibrosarcomas, rodent skin carcinoma (Gilles *et al.* 2001; Hirosaki *et al.* 2002), remodelling mammary glands and epithelial neoplasia (Koshikawa *et al.* 2000).

1.4 FIBRONECTINS

Fibronectins (FN) are glycoproteins of high molecular weight found in many extracellular matrices and in blood plasma. FN can be divided into soluble plasma FN (pFN) and insoluble cellular FN (cFN). Plasma FN is mainly synthesized in the liver by hepatocytes whereas cFN is synthesized locally by cells in the ECM. Cellular FN consists of a larger and more heterogeneous group of FN isoforms that are both cell-type-specific and species-specific (Pankov & Yamada 2002). FN can influence cell adhesion, morphology, migration, differentiation and cytoskeletal organization (Magnusson & Mosher 1998). Fibronectin is especially abundant in the ECM of embryonic and regenerating or injured tissues, although it can be found in most ECM,

including basement membranes. It interacts with cells through integrins linking the ECM to the intracellular cytoskeleton and signalling pathways (Magnusson & Mosher 1998).

1.4.1 Structure

FN exists as a soluble protomeric form in micromolar concentrations in blood plasma and as an insoluble multimeric form in the ECM (Kreis & Vale 1993). Soluble FN is a dimeric glycoprotein of two nearly identical 210-250kDa subunits linked covalently near their C-terminus by a pair of disulfide bonds (Pankov & Yamada 2002) (Figure 1.1). Each subunit of the FN molecule is made up of a series of repeating units which in turn form structural and functional domains which mediate binding to cell surface receptors or other ECM molecules. The different FN isoforms arise by alternative splicing of the transcript of a single gene (Magnusson & Mosher 1998).

Each FN subunit is made up of three types of repeating modules, type I, type II and type III. There are 12 type I modules (each around 40-45 amino acids long, clustered in 3 groups), 2 type II modules (each 60 amino acids long) and 15-17 type III modules depending on the splicing (about 90 amino acids long). Furthermore, there is a variable (V or IIICS) sequence that is not homologous to other parts of FN. Type I and II modules each contain the 2 disulphide bonds and together account for approximately 90% of the FN sequence (Pankov & Yamada 2002).

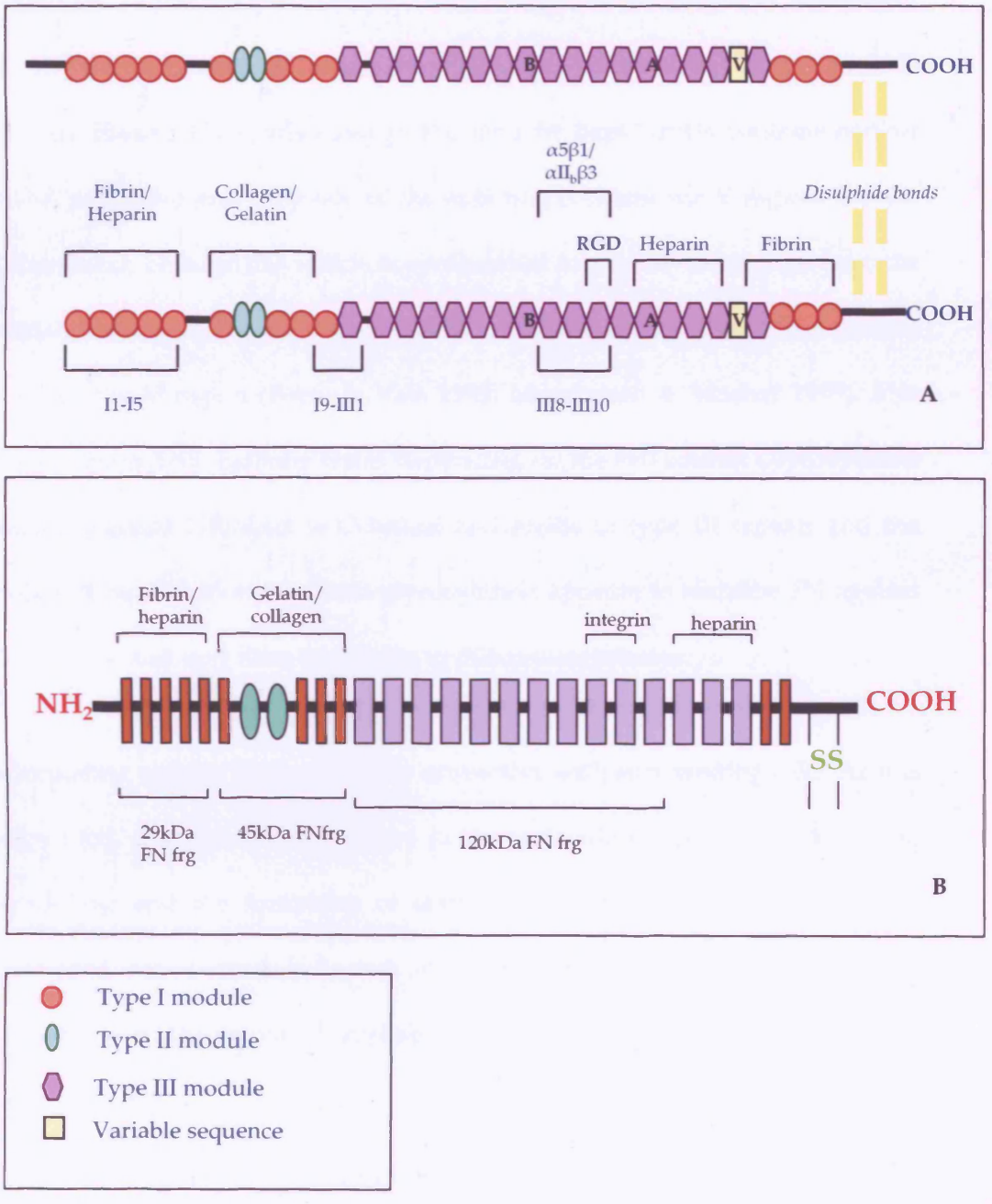


Figure 1.1 Dimeric structure of FN (A). Structure of the fragments (B).

There are 2 cysteines per subunit. The 2 FN type III modules that are subject to alternative slicing are called extradomain A (ED-A) and extradomain B (ED-B). Plasma FN synthesized in the liver by hepatocytes contains neither ED-A nor ED-B and only one of the subunits contains the V region. On the other hand, cellular FN which is synthesized locally in the tissues contains variable amounts of either or both ED-A and ED-B and almost all the subunits contain the V region (Kreis & Vale 1993; Magnusson & Mosher 1998). FNs may contain 4-9% carbohydrates depending on the cell source. Glycosylation sites are either N-linked or O-linked and reside in type III repeats and the collagen-binding domain. Such glycosylation appears to stabilise FN against hydrolysis and may alter its affinity to different substrates.

Circulating soluble FN is relatively unreactive with surrounding cells. As it is deposited, conformational changes in the molecule support cell attachment, spreading and the formation of many focal contacts with the cells. This increased adhesiveness is known as "surface activation of pFN" and can be explained by the increased availability of the RGD-containing 10th Type III repeat (Ugarova *et al.* 1995).

1.4.2 Fibronectin variants

Alternative splicing in three different regions, EDA, EDB and V give rise to up to 20 different mRNA in a cell-specific manner (Srebrow *et al.* 2002). The main type of splicing occurs in the central portion of repeat III. The exon is either

used or skipped leading to the inclusion or exclusion of 2 type III repeats, EDB between repeats III_7 -III_8, and EDA between repeats III_{11}-III_{12}. This type of ED splicing is found in many vertebrates including human. A second type of splicing involves the V or IIICS region. The structural variations in this region are more complex and species dependent. In general, this region can be either partially or completely included or excluded. In human FN there can be five different V region variants. A third type of splicing is found in cartilage where the whole V region and the FN III_{15} and FN I_{10} repeats are missing (Pankov & Yamada 2002).

Such changes in alternative splicing of the mRNAs occur as a result of stimulation by growth factors, cytokines, hormones and stress stimuli. Cell-ECM interactions initiate a dynamic flow of information that regulates many important processes throughout development. Among them is cell migration during embryogenesis, morphogenesis during organ formation, and the modulation of growth, survival and differentiation of many specialized cell types (Srebrow *et al.* 2002).

Both EDA and EDB-containing FNs are expressed predominantly during embryogenesis. Their expression is also increased during wound healing. EDA-containing FN has been associated with the regulation of cell adhesion, proliferation and differentiation. EDB-containing FN seems to have a role in angiogenesis (Liao *et al.* 2002). A study on epithelial cells showed that

migrating cells change the type of FN secreted to an EDA-containing FN despite producing lesser amounts. This clearly shows that in many cases the characteristics of the FN may be a stronger stimulus than the amount produced (Inoue *et al.* 2001).

1.4.3 Fibronectin receptors

One of the most important families of proteins that bind to FN are the integrins which mediate cell-to-FN binding and serve to trigger function-specific intra-cellular pathways. A large number of integrin heterodimers bind to FN including the classic FN receptor α5β1 integrin. FN has several integrin-recognition sequences, the best known of which is the RGD sequence in III_{10} (Pankov & Yamada 2002). RGD peptides can compete with this binding and block integrin-mediated cell adhesion to FN (Magnusson & Mosher 1998). RGD sequences occur in other molecules such as vitronectin, tenascin and TGF-β latency-associated-peptide (LAP) and these also mediate adhesions to integrin receptors (Thomas *et al.* 2002). Integrins that bind to this region include α5β1, α3β1, αvβ1, αvβ3 and αvβ6. The binding of α5β1 to FN is synergized by modules III_8-III_9, specifically the Pro-His-Ser-Arg-Asn (PHSRN) synergy sequence in III_9 (Pankov & Yamada 2002). Binding to the α5β1 integrin can also occur at the N-terminal region containing repeats I_{1-9} and $II_{1,2}$. This region can trigger intra-cellular signals different from those triggered by the interaction with the RGD sequence (Akiyama & Yamada 1995).

Several sites from the COOH-terminal to the RGD sequence interact with the cell surface. This region, known as the heparin-binding domain (HBD, III_{12-14}) can attach to heparin sulphate proteoglycans and chondroitin sulphate proteoglycans. The III_{14} also has a low affinity for the α4β1 integrin. Further towards the COOH-terminal from the HBD, along the alternatively spliced IIICS or V region are the α4β1 binding sites CS-1 and CS-5, of which the CS-1 has the higher affinity. This site is also recognized by the α4β7 integrin (Huhtala *et al.* 1995).

There are other cell-recognition sequences along the FN molecule known as LDV and REDV that bind to the α4β1 and α4β7 integrins. They are found on the alternatively spliced V region. The α4β1 integrin, as well as the α9β1 have been identified to bind to the EDA segment of FN. Since this segment of FN is alternatively spliced, it generates a novel motif for α9β1 (which finds no other binding site on the molecule) and an additional site for α4β1 (Liao *et al.* 2002; Pankov & Yamada 2002).

FN has many other adhesive sites for various substances including fibrin, heparin, collagen/gelatin which make it an important molecule not only in cell-ECM binding (Akiyama & Yamada 1995). Two major heparin-binding sites are Heparin II, which has high affinity and is located at the C-terminus, and Heparin I, with weaker affinity located at the N-terminus. Heparin II binds to chondroitin sulphate and Heparin I contains a staphylococcus-

aureus-binding domain which enables this molecule to interact with bacteria. A recently described domain in the V region which binds to glycosaminoglycans (named "Heparin" at the V-region) may potentiate cell adhesion (Pankov & Yamada 2002).

The collagen binding domains in FN are located at repeats I_{6-9} and $II_{1,2}$. As it binds more effectively to denatured collagen (gelatin) it is thought to be important in the interaction with denatured collagenous materials from blood than to mediating cell adhesion to collagen (Pankov & Yamada 2002). Fibrin I and Fibrin II are the major fibrin-binding sites in FN located at the N-terminus. This interaction with fibrin is important in the clotting mechanism of blood (Pankov & Yamada 2002).

1.4.4 Fibronectin matrix assembly

FN matrices are deposited both spatially and temporally in a defined pattern utilising both the soluble and insoluble FNs. This assembly occurs in cell-mediated co-ordinated steps, the first of which is the binding of FN to the cell surface via the interaction with the N-terminus 70 kDa region of the molecule. This cell-bound FN is converted into a stable multimer by the formation of disulphide bonds as the FN molecules interact (Hocking *et al.* 2000). In addition, the NH_2-terminal 29-kDa domain is required for assembly of a fibrillar FN matrix. It has fibrin- and weak heparin-binding activities and binds weakly to the cell surface (Huhtala *et al.* 1995).

Integrins and LAMMs (molecules of large apparent molecular mass) are integral to FN matrix assembly (Magnusson & Mosher 1998). Most of the binding activity that directs FN to assembly is in the 70-kDa region of FN. The specific integrins required in matrix assembly is dependent on the substrate to which assembly cells are adherent to. The main integrin responsible for matrix assembly is α5β1 although other FN binding integrins can initiate the process, including αvβ3 which recognises the 10th type III module, and α3β1 integrin which acts indirectly by binding first to entactin which then binds to FN (Wu *et al.* 1995; Magnusson & Mosher 1998).

Ligation of integrins on the cell surface to the ECM induces the clustering of actin and actin-binding proteins to focal adhesions and mechanically couples the ECM with the actin cytoskeleton. FN polymerization is thus critical in triggering cytoskeletal tension generation necessary for cell contractility (Hocking *et al.* 2000).

1.4.5 Fibronectin fragments

Soluble FN in its compact conformation reacts poorly with cells. However, proteolysis reveals adhesive properties that are latent in the intact molecule (Ugarova *et al.* 1995). This occurs by the exposure of various biologically active domains that are sterically hindered in the intact molecule. Fibronectin may exist as a variety of molecular weight fragments in the ECM, many possessing differing bioactivities. Elevated levels of FN fragments have been

found in various pathological conditions such as in the synovial fluids of arthritic patients and in gingival crevicular fluid in patients with periodontitis (Kapila *et al.* 1996; Huynh *et al.* 2002; Gemba *et al.* 2002). When found as peptides, the cell-binding sites of FN can function in several different ways: (a) they may mimic the intact molecule, (b) they may act as a competitive inhibitor, (c) they may augment integrin signalling and activate cell-to-cell adhesiveness in certain cell types. Bearing this in mind, some fragments may be utilised in tissue engineering and wound repair or alternatively may be used to inhibit unwanted effects such as tumour cell invasion and metastasis (Kapila *et al.* 1996; Huynh *et al.* 2002).

1.4.5.1 Cell-binding fragment

The 120kDa FN fragment contains the cell-binding region of FN (RGD). This region has shown to bind to the α5β1 integrin, and also to the α3β1 integrin. Such fragment is formed *in vivo* by the action of proteases and is manufactured commercially by the action of chymotrypsin (Akiyama & Yamada 1995). An important observation was made when comparing the effect of pre-incubating the cells with anti-α5β1 and -α3β1 antibodies as opposed to adding them to the cells after they are exposed to them. It was shown that pre-incubating the cells yielded the desired inhibitory effect whereas adding the antibodies after the cells were exposed to the fragments had little or no effect. This may be explained by the fact that once the cells are

exposed to the fragments the binding sites are fully occupied leaving no opportunity for antibodies to bind (Livant *et al.* 2000).

It has been shown that the 120kDa fragment elicits different responses compared to the intact molecule. These include increased expression of collagenase, stromelysin and the serine protease urokinase in human periodontal ligament cells (Kapila *et al.* 1996), and increased collagenase, stromelysin-1 and gelatinase expression in rabbit synovial fibroblasts (Huhtala *et al.* 1995). Other studies have shown that this cell-binding fragment increased the release of pro-stromelysin-1 and the release of urokinase-type plasminogen activator (uPA) but not the tissue-type (tPA) in cultures of bovine chondrocytes (Xie *et al.* 1994; Bewsey *et al.* 1996). In mammary epithelial cell lines the 120kDa FN fragment reduced cell numbers and induced apoptosis and matrix degrading protease activity (Schedin *et al.* 2000). In wound healing studies the cell-binding fragment stimulated monocytes and fibroblast chemotaxis through the dermis, increased the release of MMPs by fibroblasts and stimulated monocyte differentiation into inflammatory macrophages (Livant *et al.* 2000) (Table 1.2).

1.4.5.2 Amino-terminal fragment

The 29kDa amino-terminal heparin-binding FN fragment has been found to possess potent chondrolytic effects and may be implicated in the cartilage degradation of arthritis. In cartilage explant cultures it was found to elevate

MMP production, enhance rates of proteoglycan loss and suppress proteoglycan synthesis (Gemba *et al.* 2002). In cultured human articular cartilage it was also found to increase levels of tumour necrosis factor-α, interleukin-1β (IL-1β) and IL1-α (Gemba *et al.* 2002). It has also been implicated in promoting cell motility in soft tissue sarcomas (Liu *et al.* 1998) (Table 1.2).

The influence of a number of FN fragments including the 29kDa, the 50kDa gelatin-binding, the 140kDa integrin-binding fragment and the 35kDa carboxyl-terminal heparin-binding fragment on the release of prostaglandins (PG) in the synovial fluid of osteoarthritis (OA) and rheumatoid arthritis (RA) has been studied. Specific fragment combinations resulted in up to 23-fold increase in PG release (Homandberg *et al.* 1992). This 29kDa amino terminal fragment may work in synergy, with combinations producing increased response as compared to individual fragments (Homandberg *et al.* 1992).

1.4.5.3 PHSRN peptide

This peptide sequence found on repeat III_9 promotes specific α5β1 integrin binding to FN and is therefore also known as the "synergy site" (Yamada 2000; Pankov & Yamada 2002). In a study using sea urchins as in vitro invasion substrates, the PHSRN sequence of pFN was found to stimulate ECM invasion by both keratinocytes and fibroblasts (Table 1.2). This peptide, in an acetylated and amidated form was shown in this study to be more

potent than its non-acetylated counterpart and the 120kDa cell-binding FN fragment. The presence of this peptide in solution has also been shown to stimulate dissociation of adherent cells from the substratum (Livant *et al.* 2000; Yamada 2000).

1.4.5.4 EDA and EDB fragments

Previous studies indicate that the presence of EDB domain in certain cell-binding FN fragments may increase cell spreading and adhesion. This was not observed with the EDB fragment alone suggesting that it either simply provides an additional integrin binding site, or that when present, it generates conformational modifications in the other domains possibly improving access to integrin-binding sites (Hashimoto-Uoshima *et al.* 1997) (Table 1.2). Other groups have attributed this same synergistic property to the EDA domain (Chen & Culp 1996).

FRAGMENT	SIZE	EFFECT
Cell-binding fragment:	120kDa	• ↑ collagenases, stromelysin and PA production by human periodontal ligament cells (Kapila *et al.* 1996). • ↑ collagenases, stromelysin-1 and gelatinases by rabbit synovial fibroblasts (Huhtala *et al.* 1995). • ↑ release of pro-stromelysin-1 and uPA in cultures of bovine chondrocytes (Xie *et al.* 1994; Bewsey *et al.* 1996). • ↑ matrix degrading proteins and reduction in apoptosis in mammary epithelial cells (Schedin *et al.* 2000). • Stimulation of monocyte and fibroblast chemotaxis, increased MMP release by fibroblasts and stimulation of monocyte differentiation in wounds (Livant *et al.* 2000).
Amino-terminal fragment:	**29kDa**	**• ↑ MMP production, increased proteoglycans loss and suppression of proteoglycans synthesis by chondrocytes in arthritic patients (Gemba *et al.* 2002). • ↑ levels of tumour necrosis factor-α, interleukin-1β (IL-1β) and IL1-α in human articular cartilage (Gemba *et al.* 2002). • ↑ cell motility in soft tissue sarcomas (Liu *e al.* 1998).**
PHSRN synergy sequence:	Peptide	• Promotion of α5β1 binding to RGD (Yamada 2000; Pankov & Yamada 2002). • Stimulation of ECM invasion by keratinocytes and fibroblasts (Livant *et al.* 2000; Yamada 2000).
EDA/EDB domains:	**Peptides**	**• ↑ cell spreading and adhesion (Chen & Culp 1996; Hashimoto-Uoshima *et al.* 1997).**

Table 1.2 Various FN fragments and their effects.

1.5 CELL-ADHESION MOLECULES

The structure and organization of body tissues is dependent on preservation of contact between cells and their neighbours and between cells and the ECM (Garrod 1993; Pignatelli 1998). Adhesion molecules are responsible for cell stability, and contribute to their morphology, differentiation, motility and internal signalling (Eversole 1996). There are four known families of cell-adhesion molecules: the cadherins, the immunoglobulin superfamily, integrins and selectins (Garrod 1993). Mouse knock-out experiments have confirmed the necessity for cadherins and integrins in stable cell-cell and cell-matrix adhesion (Pignatelli 1998).

1.5.1 Cadherins

Cadherins are calcium-dependent cell adhesion molecules responsible for cell-to-cell adhesion (Pecina-Slaus 2003). They play a key role in calcium-dependent cell-cell interactions and act not only to establish tight cell-cell adhesion but also to define adhesive properties of cells (Wheelock & Johnson 2003). Cadherins constitute a large family of transmembrane glycoproteins that can be divided into subfamilies based on molecular features (Alberts *et al.* 1994; Wheelock & Johnson 2003). The classical cadherins expressed by keratinocytes are E- and P-cadherins (Bagutti *et al.* 1998). P-cadherin, along with integrins is expressed predominantly in the basal layer of stratified epithelium, while E-cadherin is expressed in all living cell layers (Bagutti *et al.*

1998). During development the expression of each subtype is spatiotemporally regulated so as to be correlated with morphogenetic events during cell adhesion processes. They are therefore important in cell recognition, adhesion, sorting and signalling. Disruption of cadherin function has serious and significant implications in disease conditions including cancer (Wheelock & Johnson 2003).

The epithelial cadherin E-cadherin (also known as uvomorulin) appears very early in development and is the best characterized cadherin (Garrod 1993). E-cadherins may be found clustered in continuous adhesion belts, known as zonulae adherens, which encircle the cell apicolaterally. Such adhesion belts are held together by interacting extra-cellular domains of E-cadherins. E-cadherins may also be found grouped in desmosomes, buttonlike points of intercellular contacts (Alberts *et al.* 1994). Cadherins bind by a homophilic mechanism, where a cadherin molecule on one cell binds to a cadherin molecule on another (Garrod 1993). Recently however, E-cadherin has also shown to be a ligand for two integrins, αEβ7 and α2β1; the former is important in the retention of intraepithelial lymphocytes in mucosal tissue while the latter helps contribute to the organization of epithelial multilayers (Pecina-Slaus 2003).

1.5.1.1 Structure of E-cadherin

Cadherins constitute a large family of transmembrane glycoproteins composed of about 700-750 amino acid residues (Alberts *et al.* 1994; Wheelock & Johnson 2003). Proteins are considered members of the cadherin family if they have one or more cadherin repeats; these repeats are independently folding conserved sequences of approximately 110 amino acids (Behrens 1999; Pecina-Slaus 2003; Wheelock & Johnson 2003). Calcium is essential for the optimal adhesive function of E-cadherins and their protection against protease digestion. The calcium ions bind to specific residues in each cadherin repeat to warrant its proper folding and to give rigidity upon the extracellular domain (Guilford 1999; Wheelock & Johnson 2003). The intracellular domain of the molecule is linked to the cell cytoskeleton via the intracellular attachment proteins α-, either β- or γ- catenins, vinculin, α-actinin and plakoglobin (Garrod 1993; Pignatelli 1998; Pecina-Slaus 2003) (Figure 1.2). Beta and γ- catenins share homology and bind to a specific domain at the E-cadherin C-terminus; the α-catenins then links the bound β- or γ-catenin to the actin cytoskeleton (Guilford 1999; Pecina-Slaus 2003). In this way, an extensive trancellular network is formed (Alberts *et al.* 1994). In the absence of catenins, cadherins are unable to function (Garrod 1993; Alberts *et al.* 1994).

1.5.1.2 E-Cadherin and tissue development

During embryogenesis, tissues require the coordination of cellular processes, including polarization, adhesion, aggregation, segregation, differentiation

and migration (Pignatelli 1998; Guilford 1999; Pecina-Slaus 2003; Wheelock & Johnson 2003). The expression of specific adhesion proteins is vital not only to normal development but also for the maintenance of normal tissue structure and integrity (Guilford 1999; Wheelock & Johnson 2003). Different cadherin family members are expressed in specific spatiotemporal patterns during development and are important regulators of morphogenesis and tissue formation (Behrens 1999; Wheelock & Johnson 2003).

The establishment of cellular polarity occurs in the earliest stages of development and is mediated by E-cadherin. Furthermore, E-cadherin constitutes a major cellular adhesion/recognition system that grants cells the ability to sort from one another. When a group of cells separates from an existing cell layer the segregating cells express qualitatively or quantitatively altered sets of cadherins. This may then turn off or on some cadherin genes (Wheelock & Johnson 2003). Such gene silencing in E-cadherin expression has been associated with hypermethylation of the CpG islands, found in promoter regions of regulatory genes in both breast and prostate carcinomas (Graff *et al.* 1995; Auerkari 2005).

1.5.1.3 E-cadherin in cancer

Decreased expression of E-cadherin is seen as one of the main molecular events involved in cell-cell adhesion system dysfunction, triggering cancer invasion and metastasis (Pecina-Slaus 2003). Many studies have shown E-

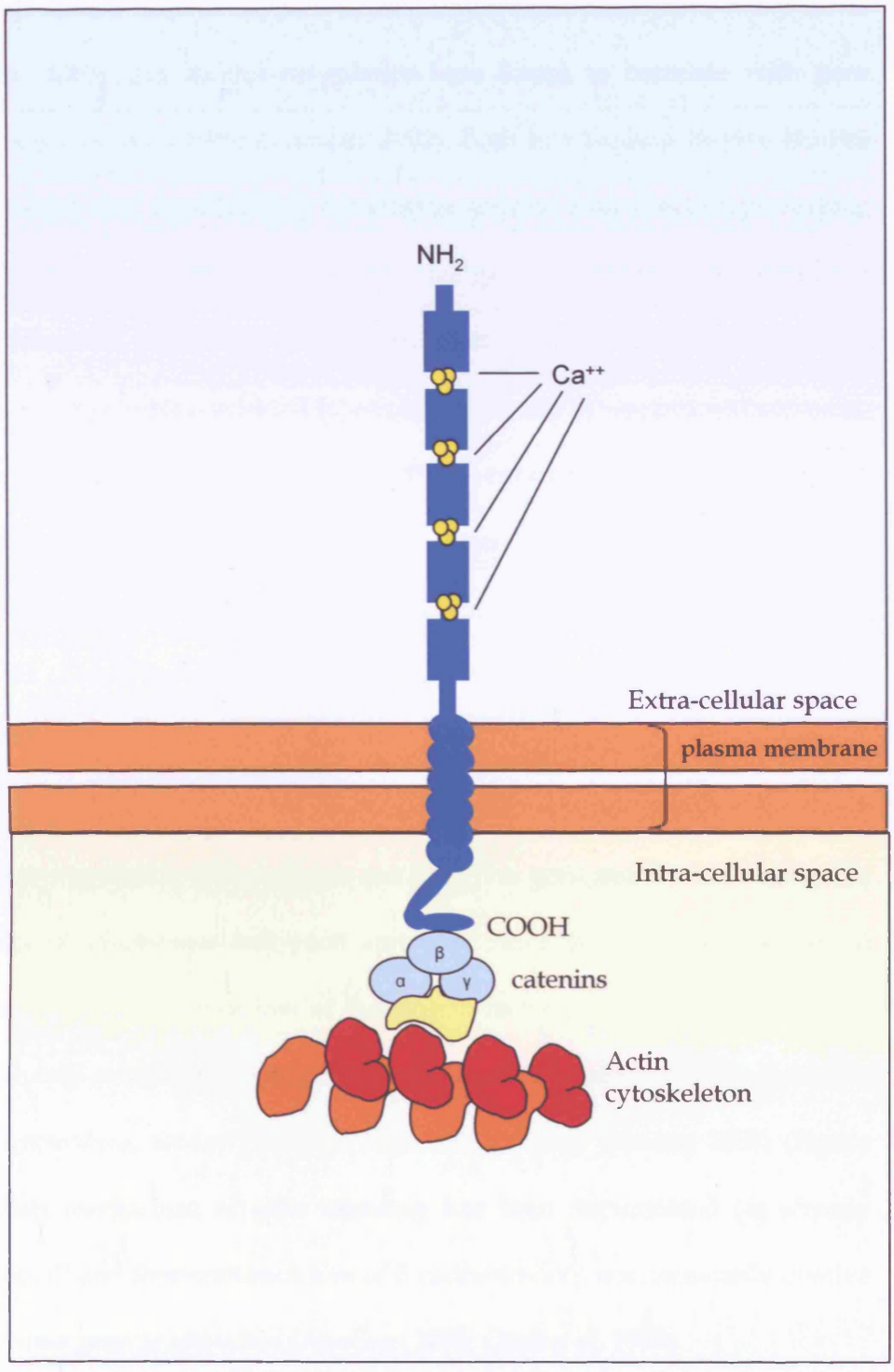


Figure 1.2 The structure of cadherins (taken after Alberts *et al.* 1994).

cadherin to be a tumour suppressor molecule (Pecina-Slaus 2003; Wheelock & Johnson 2003) and its downregulation was found to correlate with poor prognosis (Guilford 1999; Quaranta 2002). Both in vitro and in vivo studies have shown that by inhibiting E-cadherin activity with function-perturbing antibodies normal epithelial cells were changed into invasive ones (Guilford 1999; Wheelock & Johnson 2003). Conversely, invasive E-cadherin negative carcinoma cells were converted to non-invasive cells by exogenous expression of E-cadherin (Wheelock & Johnson 2003). As epithelial cells progress through the process of carcinogenesis, they change from an epithelial to a mesenchymal phenotype exhibiting a more motile and dynamic character. A hallmark of such transition is the decreased expression of E-cadherin (Behrens 1999; Wheelock & Johnson 2003).

The downregulation of E-cadherin can occur via gene mutation; however the majority of carcinomas lack such mutation. Since by immunohistochemical studies there is apparent loss of E-cadherin in most tumours, it seems that tumour cells must downregulate the activity of E-cadherin in order to invade the surrounding tissues (Behrens 1999; Wheelock & Johnson 2003) (Figure 1.3). This mechanism of gene silencing has been documented (as already mentioned) and therefore such loss of E-cadherin does not necessarily involve irreversible genetic alteration (Auerkari 2005; Graff *et al.* 1995).

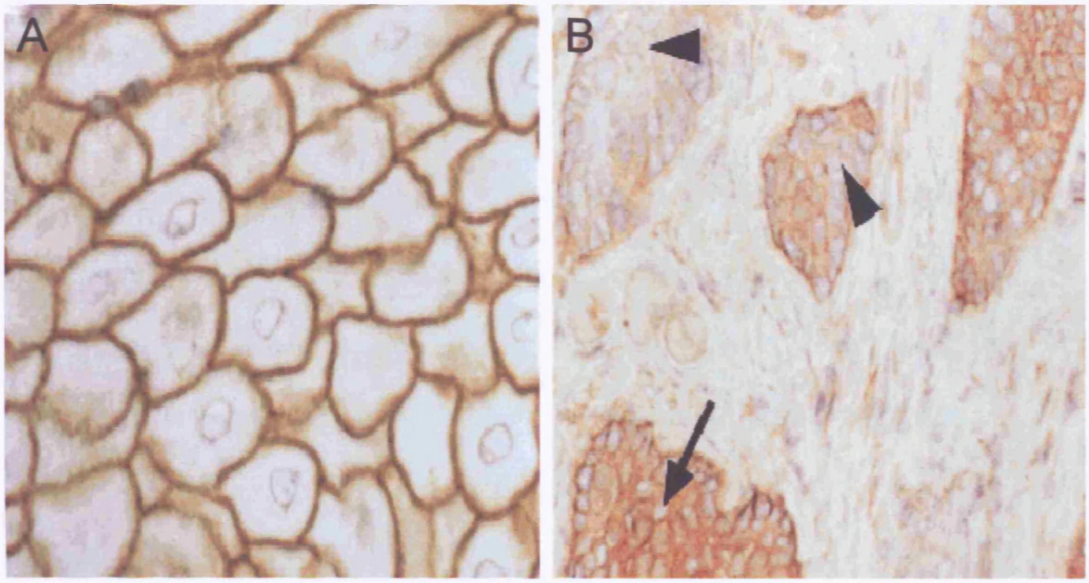


Figure 1.3 E-cadherin expression in normal and malignant oral epithelium. Cells exhibit continuous E-cadherin expression in normal epithelium (A). Epithelial tumour nests showing areas of discontinued E-cadherin expression (short arrows) and areas of persistent E-cadherin expression (longer arrow) denoting some down-regulation of this cell-adhesion molecules in carcinomas (B) (Bagutti *et al.* 1998).

Alternatively, E-cadherin function can be disrupted by upsetting the connection between the cadherin and the cytoskeleton. For example, mutations in β-catenin disturb the binding to α-catenin resulting in a non-adhesive phenotype. Mutations in the gene that encodes α-catenin inactivate E-cadherin function by not allowing the cadherin complex to associate with the cytoskeleton (Wheelock & Johnson 2003).

Some studies have demonstrated the reappearance of E-cadherin expression in metastatic cells. Such reestablishment of cellular contact may prevent apoptosis and enhance tumour cell adhesion at the metastatic site (Guilford 1999) therefore guaranteeing the tumour's immortality (Pecina-Slaus 2003).

1.5.2 Integrins

Integrins are one of the largest families of cell-adhesion molecules providing attachment between the cell and the surrounding ECM. They are transmembrane proteins that connect the ECM to the cell's cortical cytoskeleton and trigger numerous intra-cellular pathways (Alberts *et al.* 1994). They mediate cell adhesion, spreading, migration, proliferation and differentiation (Alberts *et al.* 1994; Hynes 2002). It has been shown that the composition and organization of the ECM can regulate the expression of genes and this is thought to occur through integrin engagement (Huhtala *et al.* 1995; Aplin *et al.* 1998). Integrin expression is altered in tumours and

expression varies both between different tumours and in different regions in the same tumour (Bagutti *et al.* 1998).

1.5.2.1 Structure and function

Integrins are heterodimers made up of two noncovalently associated transmembrane glycoprotein subunits called α and β, both of which contribute to the binding of the matrix protein (Thomas *et al.* 1997; Aplin *et al.* 1998; Giancotti & Rouslahti 1999). Both subunits consist of a large extra-cellular domain, a transmembrane domain and a short cytoplasmic domain (Thomas *et al.* 1997). The α chain contains several cation binding regions and is usually quite long varying between 120-180kDa (Hynes 1992) (Figure 1.4). The binding of integrins to their ligands depends on extracellular divalent cations, some requiring Mg^{++} and others requiring Ca^{++} (Alberts *et al.* 1994; Thomas *et al.* 1997). All integrins except α6β4 integrin bind to the actin-based microfilament system (Hynes 2002). The cytoplasmic tail of the β subunit binds intracellularly to both talin and α-actinin and in so doing initiates the assembly of a complex of intracellular attachment proteins that link the integrin to actin filaments in the cell cortex. If the β-chain were missing, the integrin would be able to retain some ability to bind to the ECM but would loose its ability to trigger cellular responses (Hynes 2002). This communication is bi-directional; the ECM binding may influence the organization of the cell's cytoskeleton and the cell's cytoskeleton may in turn influence the orientation of secreted ECM proteins (Alberts *et al.* 1994). The β4

subunit differs from the others in the size of the cytoplasmic domain; being much larger it interacts with the intermediate filaments instead of actin (Hynes 2002).

To date 8-β and 18-α subunits have been described combining to form 25 distinct integrins (Hynes 2002) (Figure 1.5). Integrins recognize short peptide motifs, such as RGD on ECM proteins. Some integrins bind to just one substrate and others have the ability to bind to several. Furthermore, integrin receptors often exhibit overlap in their ligand-binding specificity (Aplin *et al.* 1998; Boudreau & Jones 1999).

1.5.2.2 Integrin activation

Most integrins are not constantly active; they can be expressed on cell surfaces in an inactive state in which they do not bind to ECM ligands or generate an intracellular signal (for example integrins on cell surfaces of circulating platelets and white cells) (Hynes 2002). But such tight regulation is not exclusive to blood cells; integrin activation is essential for normal development during processes such as cell migration, proliferation and ECM assembly (Calderwood 2004). Many cells may regulate integrin activity in a temporal and spatial fashion (Hynes 2002).

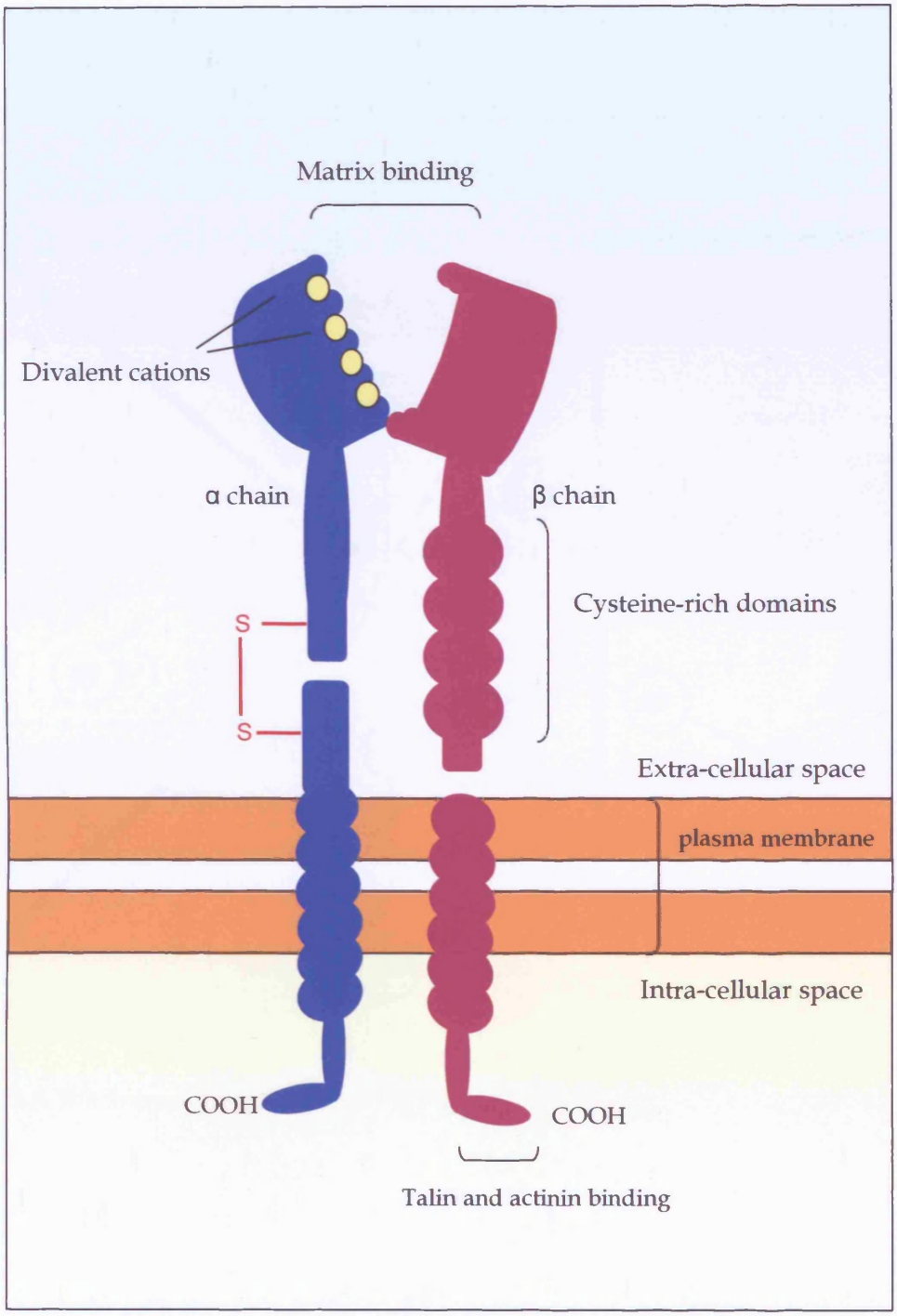


Figure 1.4 Structure of integrins (taken after Alberts *et al.* 1994).

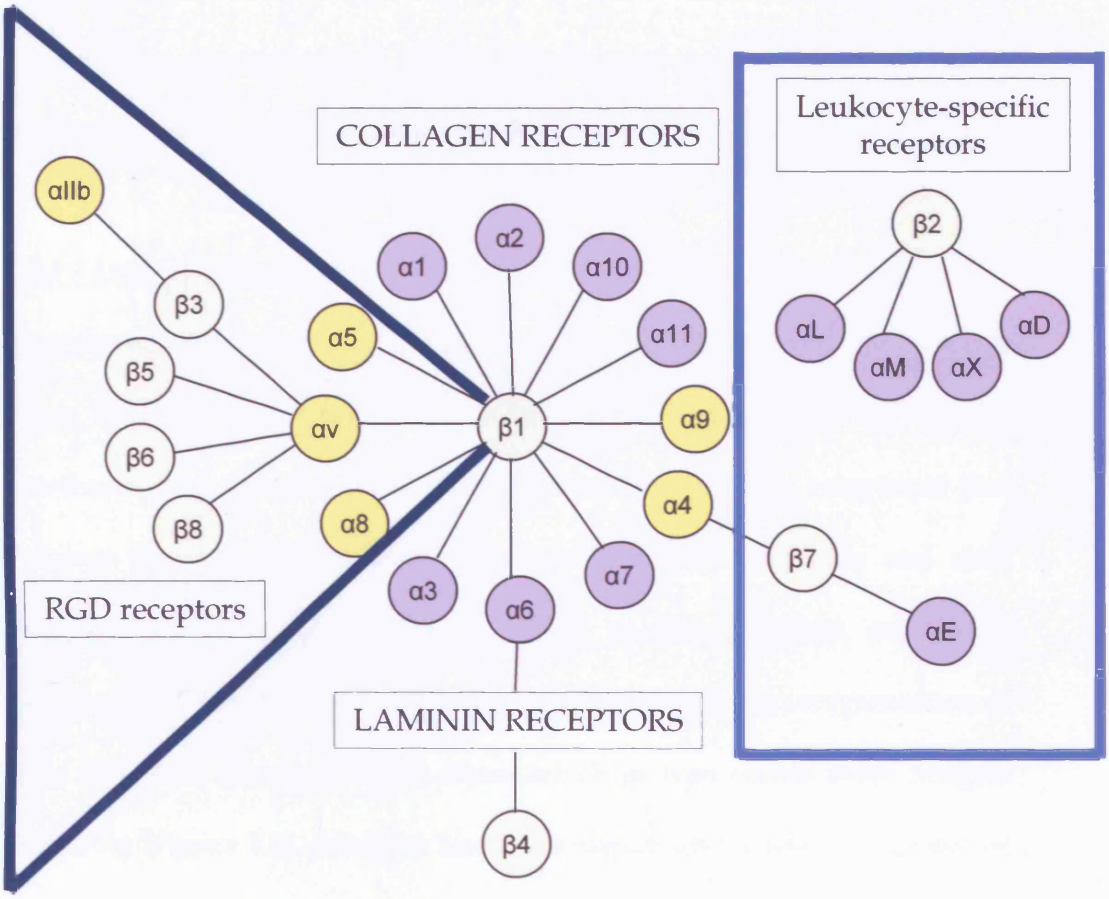


Figure 1.5 The known integrins

Integrin activation is achieved by rapid, reversible changes in the conformation of the extracellular domains of the integrin structure (Calderwood 2004). Membrane-proximal regions of both α and β tails contain a much conserved sequence, the deletion of which leads to integrin activation. Furthermore, deletion of both α and β membrane-proximal regions also leads to integrin activation (Calderwood 2004).

1.5.2.3 Integrin signalling

Integrins act as integrators of the ECM and cytoskeleton of cells (Boudreau & Jones 1999); as they bind to the ECM they cluster in a site on the cell membrane and associate with cytoskeletal and signalling complexes that enhance the assembly of actin filaments or keratin filaments and their associated proteins namely vinculin, talin, paxillin, α-actinin and tensin (Aplin *et al.* 1998; Boudreau & Jones 1999). This causes the reorganization of actin filaments into large stress fibres which in turn causes more integrin clustering (Figure 1.6). Integrins transduce signals and activate a number of signalling pathways including the non-receptor protein tyrosine kinase (PTK), mitogen-activated protein kinase (MAPK), Rho family of small guanine nucleotide binding proteins and protein kinase C (PKC) (Boudreau & Jones 1999; Giancotti & Ruoslahti 1999). Such signalling pathways then lead to alterations in cell shape, gene expression and cell behaviour (Boudreau & Jones 1999).

Focal Adhesion Kinase (FAK) is a member of the PTK family and is found to co-localize with ECM-bound integrins to induce integrin clustering(Aplin *et al.* 1998; Giancotti & Rouslahti 1999) (Figure 1.6). It plays a critical role in integrin-mediated signalling and is a common theme in multiple signal transduction pathways (Aplin *et al.* 1998). FAK tyrosine phosphorylation in reaction to cellular adhesion to ECM proteins has been shown in a variety of cells (Aplin *et al.* 1998). It interacts directly or through the cytoskeletal proteins talin and paxillin with the cytoplasmic tail of the integrin β subunit (Giancotti & Ruoslahti 1999). In addition to integrin-mediated events, FAK tyrosine phosphorylation is increased through non-integrin cell surface receptors including growth factor receptor tyrosine kinases and G-protein-coupled receptors (Aplin *et al.* 1998).

Activation of FAK is achieved when the Tyr-397 site is exposed to phosphorylation and this happens during the association of the N-terminal end of FAK with the tail of the β subunit of the integrins (Schlaepfer & Hunter 1998). FAK is unable to phosphorylate other substrates directly but integrin-induced autophosphorylation of FAK enables it to interact with docking/adaptor proteins such as paxillin, tensin, Grb2/Son of Sevenless (SOS) which in turn activate downstream signalling mediators including Src, Ras and Raf (Aplin *et al.* 1998; Schlaepfer & Hunter 1998; Boudreau & Jones 1999) (Figure 1.7). Generally, FAK activation is associated with signals that regulate growth, cell transformation and suppression of apoptosis

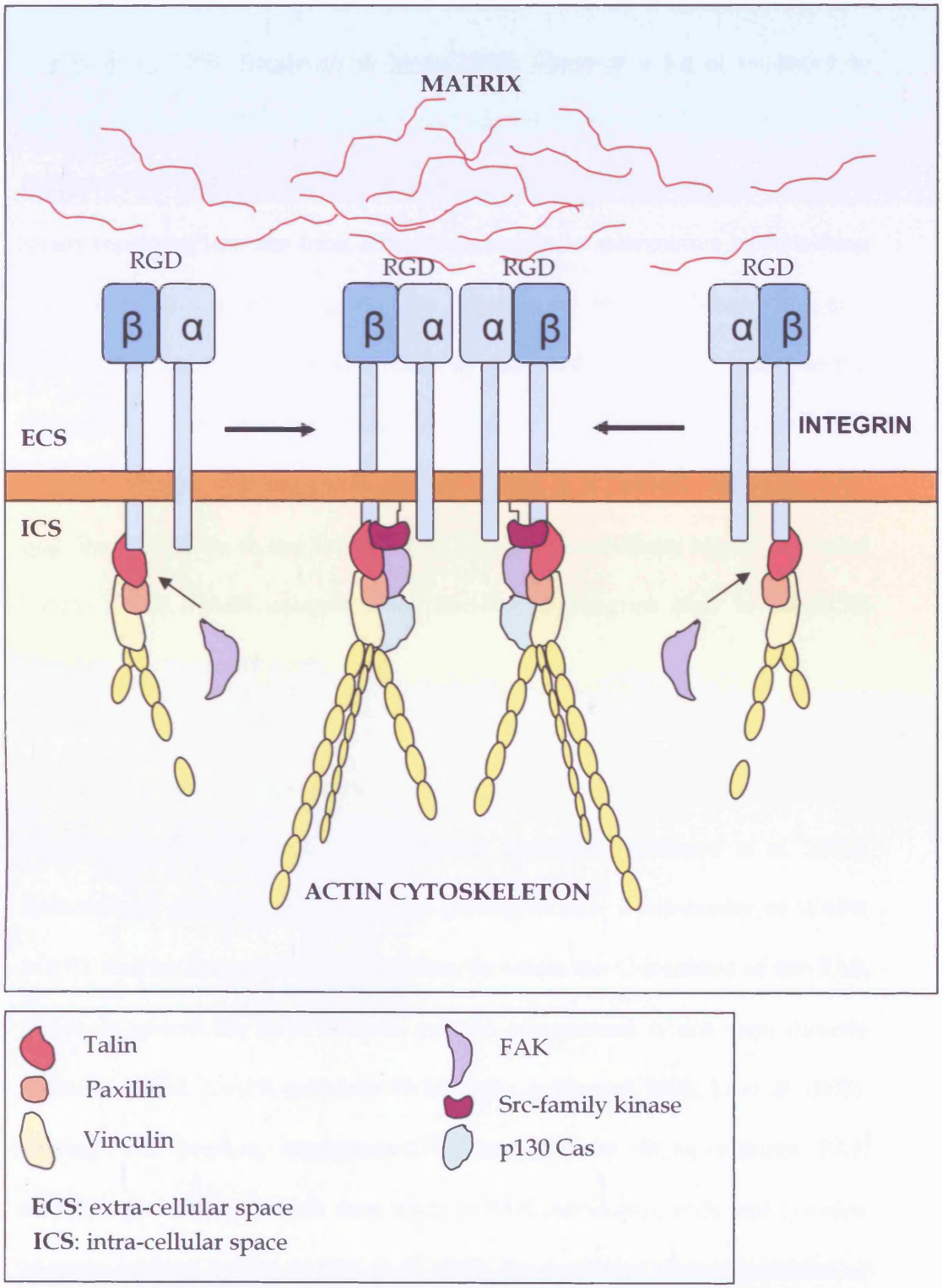


Figure 1.6 Matrix binding advocates integrin clustering and association with the cytoskeleton (taken after Giancotti & Ruoslahti 1999). As integrins cluster in response to interactions with the ECM, FAK is recruited and signalling is initiated via the actin cytoskeleton.

(Aplin *et al.* 1998; Boudreau & Jones 1999). There is a lot of evidence to suggest the convergence of integrin and growth factor signalling pathways at the level of FAK (Boudreau & Jones 1999) seen by the recruitment of growth factor receptors into the focal adhesion complexes. Alternative to activating FAK some β1 and αv integrins also activate the tyrosine kinase Fyn and through it the adapter protein Shc. In this pathway, rather than acting through talin and paxillin, caveolin-1 seems to function as a membrane adapter coupling the integrin α subunit to Fyn. It is probable that both FAK and Shc contribute to the activation of the Ras-extracellular signal-regulated kinase (ERK) MAPK cascade when Shc-linked integrins bind to the ECM (Giancotti & Ruoslahti 1999).

MAP kinases act as convergence point for various receptor-initiated growth-signalling events initiated at the plasma membrane (Ahmed *et al.* 2002). Extracellular signal-regulated kinases (ERKs) include a sub-family of MAPs. MAPK can be activated by FAK indirectly when the C-terminal of the FAK molecule causes the Grb2 adaptor protein recruitment which then directly activates ERK2/MAPK pathway (Schlaepfer & Hunter 1998; Li *et al.* 2003). During this process, engagement of integrins by ECM induces FAK autophosphorylation which then leads to FAK association with and tyrosine phosphorylation by Src (Aplin *et al.* 1998). Scr-mediated phosphorylation of FAK encourages the direct binding of Grb2 adapter protein to Tyr925 of FAK. This may also promote the formation of a FAK-Grb2/Shc complex which is

independent of Tyr925 phosphorylation. However, both mechanisms result in the recruitment of SOS to the juxtamembrane complex and the subsequent activation of Ras which then activates Raf (Aplin *et al.* 1998). Integrin-activated ERK translocates to the nucleus and activates transcription factors such as Elk1 and c-Myc. This activation induces gene-related responses to ECM adhesion binding. Such integrin-mediated signalling acts in synergy with growth factor signalling in mediating DNA changes at the level of MAP kinases (Schlaepfer & Hunter 1998).

Which of the pathways predominates in contribution depends on cell type and maybe on how far the adhesion process has progressed. In many cell types Shc seems to be in charge of the initial high-level activation of ERK upon cell adhesion. The more slowly activated FAK may then sustain ERK activation (Giancotti & Ruoslahti 1999).

Many integrin-stimulated intracellular signalling pathways are similar to those triggered by growth factor receptors and are very closely coupled with them. Integrin aggregation and association with the cytoskeleton appear to give rise to integrin-growth factor receptor complexes (Giancotti & Ruoslahti 1999). In order for the cells to respond to growth factors such as epithelial growth factor (EGF), platelet derived growth factor (PDGF) and thrombin, they need to be adherent to a substrate via integrins. Not only does this result in the activation or near-activation of growth receptors, but also enables cross-

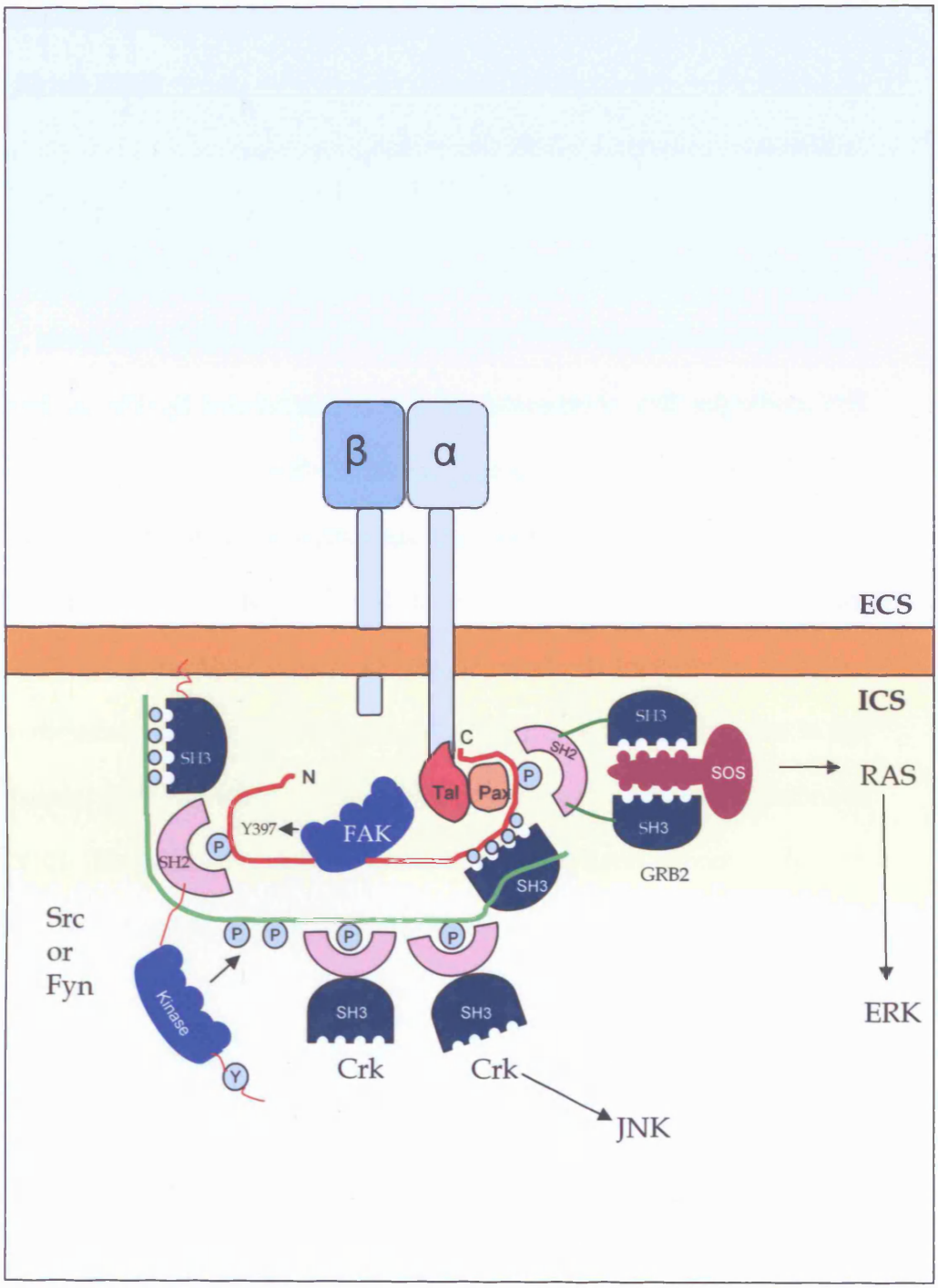


Figure 1.7 FAK pathway via α chain of the integrin. FAK is phosphorylated by its association with integrins, enabling it to interact with docking/adaptor proteins such as paxillin, tensin, Grb2/SOS, which in turn activate downstream signalling mediators such as Scr, RAS, JNK (taken after Giancotti & Ruoslahti 1999).

talk between integrins and growth factor receptors (Giancotti & Ruoslahti 1999; Hynes 2002).

1.5.2.4 Epithelial integrins

The main integrins expressed by the oral epithelium are those of the β1 family, along with α6β4 and αvβ5 (Thomas *et al.* 1997). Epithelial integrins are involved in cell-cell interactions, cell-ECM interactions, cell migration, cell stratification and terminal differentiation (Thomas *et al.* 1997) (Table 1.3). In normal human epithelium integrins are expressed very strongly by the basal keratinocytes but may also be found in the suprabasal layers. Integrins are expressed in a discrete polarized manner being situated mostly in an apico-lateral distribution. The α6β4 integrin is localized in hemidesmosomes in the basal aspect of basal keratinocytes and binds to Laminin-1 and -5 (Zambruno *et al.* 1995). The α2β1 and α3β1 integrins on the other hand appear to mediate cell-cell contact as they are found on the lateral aspects of cells.

Integrin expression is changed in malignant cells compared to their normal counterparts (Thomas *et al.* 1997). In malignant melanomas the αvβ3 integrin is expressed and is found to modulate expression of proteolytic enzymes by the tumour cells (Thomas *et al.* 1997). Most β1 integrins are expressed in elevated amounts during wound healing, hyperproliferative disease and carcinomas (Zambruno *et al.* 1995). Carcinomas exhibit variable loss of expression of α3β1, α2β1 and α5β1 (Thomas *et al.* 1997). Such altered integrin

expression seems to be involved in several aspects of neoplastic growth, invasion and metastasis. The expression of the α6β4 integrin is increased in some tumours such as squamous, bladder and gastric carcinoma, maintained in breast and colorectal tumours and newly expressed in thyroid carcinomas. However, the localization changes from the polarized hemidesmosomes, to actin-association at the leading edge under lamellopodia and filipodia (Mercurio & Rabinovitz 2001). The loss or increased expression of integrins may physically modify the adhesive properties of cells as well as rendering them unresponsive to the regulatory signals transmitted through the receptor. These alterations may lead to uncontrolled growth, proliferation and migration (Thomas *et al.* 1997).

INTEGRINS:	LIGANDS:
α2β1	Collagen type I and IV, Laminin
α3β1	**Laminin-1 and -5, Collagens, Fibronectin**
α5β1	Fibronectin
α6β4	**Laminin-1 and -5**
αvβ5	Vitronectin
αvβ6	**Fibronectin, Tenascin, Vitronectin and Latent TGF-β**

Table 1.3 Epithelial integrins and their ligands (Thomas *et al.* 1997; Li *et al.* 2003).

1.5.2.5. The α5β1 integrin

This epithelial integrin is not always detectable in healthy adult epidermal cells (Zambruno *et al.* 1995). However, it becomes highly expressed on the

surfaces of keratinocytes during active wound healing, in cell culture and in carcinomas (Zambruno *et al.* 1995). The α5β1 is a known FN receptor, recognizing the RGD sequence, and is particularly important in epithelial migration (Livant *et al.* 2000). Studies suggest that the 120kDa FN fragment-α5β1 interaction provides an inductive signal for MMP expression; this may be overridden by additional signal(s) arising from the interactions of domain(s) of FN outside the central cell-binding region (Huhtala *et al.* 1995). This integrin has been closely linked to tumour growth factor-β1 (TGF-β1). During wound healing, TGF-β1 strongly upregulates α5β1 integrins and promotes keratinocyte migration (Zambruno *et al.* 1995). This could be achieved through FN binding. This increased expression of the α5β1 integrin during wound healing could classify it as an emergency receptor that enhances cell migration and aids in the healing process (Zambruno *et al.* 1995) (See the wound healing and keratinocytes migration section).

1.5.2.6 The avβ6 integrin

The αvβ6 integrin is a receptor for FN that is not detectable on keratinocytes under normal conditions. It is increased significantly in wound healing, oral epithelial dysplasia and oral squamous cell carcinoma (Huang *et al.* 1998; Li *et al.* 2003). In such conditions the αvβ6 integrin is expressed at the leading edge of wound sections and at the interface between tumour cells and adjacent stroma (Huang *et al.* 1998). Its expression is maximal in the wound healing process when the migrating edges of the lesion have joined and correlates

with the time when FN and tenascin are actively synthesized (Clark *et al.* 1996; Haapasalmi *et al.* 1996). Several studies have shown that high expression of αvβ6 integrin correlates with the acquisition of a more malignant phenotype with increased cell migration and cell invasion (Thomas *et al.* 2001). This migration is enhanced on FN and tenascin (Huang *et al.* 1998; Thomas *et al.* 2002). Other studies have suggested that matrix modifying proteases are upregulated by keratinocytes plated on FN in an αvβ6 dependent manner (Agrez *et al.* 1994; Thomas *et al.* 2001a). It seems that αvβ6 does not affect the ability of the cells to adhere to FN, but rather increases cell migration on FN and cell invasion through reconstituted basement membrane preparations (matrigel). Recent studies have scrutinized the relation between αvβ6 and TGF-β1 and 3. Mature TGF-β remains associated with it latency-associated-peptide (LAP) and the αvβ6 integrin interacts with both TGF-β1/LAP and TGF-β3/LAP via the RGD sequence (Thomas *et al.* 2002). All these studies conclude that the αvβ6 integrin in oral squamous cell carcinoma is associated with a more malignant phenotype, characterized by increased invasiveness and metastasis (Li *et al.* 2003).

1.5.2.7 Fibronectin and avβ6 integrin in tumours

When the αvβ6 integrin couples with FN it activates the Fyn signalling pathway (Li *et al.* 2003). Once the β6 associates with Fyn, FAK is recruited and a functional complex is formed. This in turn activates Shc and links it to the Raf-ERK/MAPK pathway implicated with increased expression of MMPs (Li

et al. 2003). Activation of the Src family kinase Fyn promotes oral SCC cell proliferation and metastasis (Li *et al.* 2003).

Expression of αvβ6 integrin promotes cell migration on FN rather than adhesion to FN. Since interaction of the αvβ6 integrin with FN-mediated Fyn activation occurs simultaneously with cell migration, it seems that SCC cells initially adhere to FN using other FN receptors such as α5β1 or αvβ1. Such adhesion within 60 minutes does not activate Fyn, however, when SCC cells remain adherent to FN β6 is activated and commences the cascade of signalling that promotes cell migration (Li *et al.* 2003).

1.6 PROTEASES

A regulated turnover of ECM is essential for normal biological processes such as development. The molecules of the ECM can be broken-down by extra-cellular proteolytic enzymes that are secreted locally by the surrounding cells. Furthermore, localized degradation of the ECM is essential for cells to migrate during wound healing and in cancer (Vassalli & Pepper 1994).

There is a delicate balance between proteases and their inhibitors which creates an equilibrium of production and degradation of ECM components. During development, wound healing and during tumourigenesis this balance

may be disrupted leading to more breakdown than production. This is particularly important with regard to tumours as proteases give cancers some of their defining characteristics. However, evidence suggests that most of the increased protease production associated with malignant tumours comes from the host stroma rather than from the cancer cells themselves (Edwards & Murphy 1998). Tumour cell invasion consists of three main steps: 1)- attachment of tumour cells to the basement membrane, 2)- localized proteolytic degradation of the basement membrane and 3)- migration of tumour cells through the generated defects (Stetler-Stevenson & Yu 2001; Itoh & Nagase 2002) (Figure 1.8). The serine protease plasminogen activator/plasmin system and the matrix metalloproteinases (MMPs) are the two protease systems thought to be the most important in ECM modification. In tumours, interaction between the cancer cells and their surrounding stroma regulate the production of these proteases (Vassalli & Pepper 1994; Edwards & Murphy 1998).

1.6.1 Matrix metalloproteinases

MMPs are thought to play an important role in embryonic development, morphogenesis and tissue remodelling. However, excessive or inappropriate expression of MMPs may contribute to the pathogenesis of many tissue destructive processes including arthritis, multiple sclerosis and cardiovascular disease and is essential for growth, invasion and metastasis of malignant tumours (Shapiro 1998; Reunanen & Kähäri 2002).

Degradation of the ECM is the crucial first step in the mechanism of tumour cell invasion and MMPs are thought to be involved in this process (Stetler-Stevenson & Yu 2001). In addition, MMPs may directly influence metastatic tumour growth (Stetler-Stevenson & Yu 2001). However, MMPs not only break-down ECM molecules through proteolysis, but also expose cryptic sites of ECM proteins which may stimulate specific biological activities (Itoh & Nagase 2002). Their activity is in turn regulated by tissue inhibitors of metalloproteinases (TIMPs) and plasma inhibitor α_2-macroglobulin (Itoh & Nagase 2002).

1.6.1.1 Types of matrix metalloproteinases

MMPs are a large family of zinc-containing metalloenzymes that are able to cleave most ECM proteins (Nagase & Woessner 1999; Stetler-Stevenson & Yu 2001). They can be subdivided into two main groups: soluble MMPs and membrane-type MMPs (MT-MMPs) (Table 1.4). The soluble MMPs can be subdivided according to substrate specificity into the interstitial collagenases, gelatinases, stromelysins and metalloelastases (Itoh & Nagase 2002).

Collagenases: Collagens type I, II, III and IV are cleaved by collagenases-1 (MMP-1), collagenases-2 (MMP-8) and collagenases-3 (MMP-13). Collagenase-1 is secreted during embryonic development, wound repair and tumours, and is the only MMP unable to cleave the basement membrane. It is secreted by basal keratinocytes and is pivotal in keratinocytes migration on type I

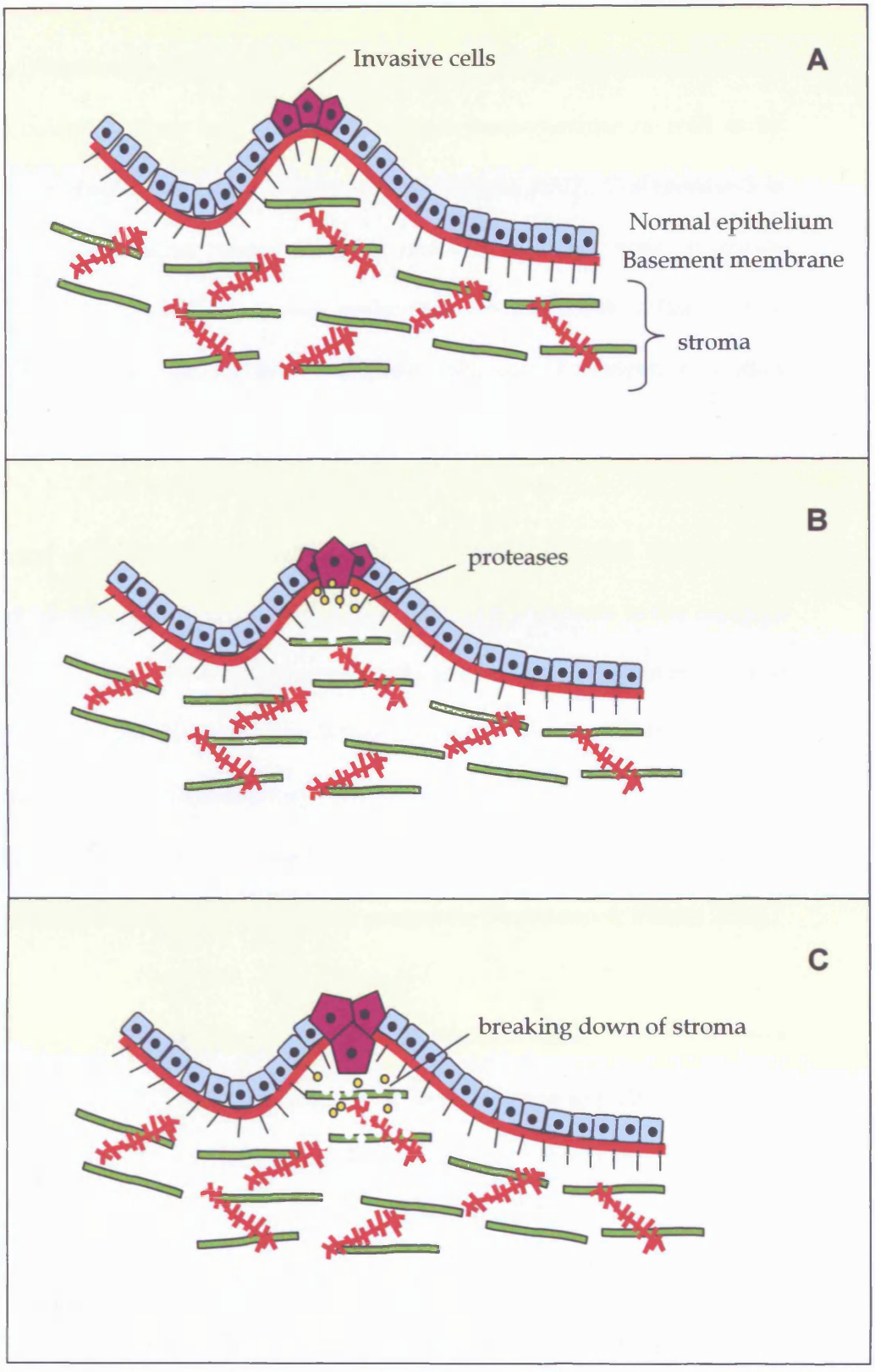


Figure 1.8 Steps in cellular invasion: A- Attachment, B- Proteolysis and C- Invasion (taken after Stetler-Stevenson & Yu 2001).

collagen (Reunanen & Kähäri 2002). Collagenase-2 is synthesized and stored by polymorphonuclear leukocytes maturing in bone marrow as well as by articular cartilage chondrocytes (Reunanen & Kähäri 2002). Collagenase-3 is expressed when rapid remodelling is needed and has wide substrate specificity. It is expressed during embryonic development, inflammatory conditions, wound healing and malignant tumours (Reunanen & Kähäri 2002).

Gelatinases: Gelatinase-A (MMP-2, 72-kDa gelatinase) and Gelatinase-B (MMP-9, 92-kDa gelatinase) contain three FN-type II repeats in the catalytic domain and are molecules that cleave components of the basement membrane and are important in conditions when basement membrane disruption is necessary, such as inflammatory conditions where white blood cells are required. They are also expressed in elevated amounts in malignancies and correlate with tumour spread and poor prognosis (Reunanen & Kähäri 2002).

Stromelysins and stromelysin-like MMPs: Stromelysin-1 (MMP-3) and stromelysin-2 (MMP-10) are expressed by keratinocytes and fibroblasts and both have a wide spectrum of substrate specificity. MMP-3 is expressed by stromal cells during mammary gland development and is an inducer of apoptosis and proliferation depending on the differentiation status of the cell. It also activates latent MMP-1. Stromelysin-3 (MMP-11) is expressed in invasive tumours and correlated with poor prognosis in breast cancer cases. It

is secreted by fibroblasts. Macrophages continually express macrophage metalloelastase (MMP-12) during pathologies such as atherosclerotic lesions, as well as by tumour cells in cutaneous SCCs (Reunanen & Kähäri 2002).

Matrilysins: The smallest MMPs are matrilysin and matrilysin-2 (MMP-7 and MMP-26 respectively), lacking the hinge region and hemopexin domain. They have wide substrate specificity and are secreted by many epithelial cell types (Reunanen & Kähäri 2002).

Membrane-type MMPs: Membrane-type MMPs are bound to the membrane by a transmembrane domain near the C-terminus except for MT4-MMP and MT6-MMP which are anchored by glycosylphosphatidylinositol (GPI)-anchor. There are six members in this group; MT1-MMP (MMP-14), MT2-MMP (MMP-15), MT3-MMP (MMP-16), MT4-MMP (MMP-17), MT-5-MMP (MMP-24) and MT6-MMP (MMP-25). Membrane-type MMPs play a role in cell-matrix interactions and cell invasion. MT1-, MT3-, MT5- and MT6-MMPs activate pro-MMP2 and MT1- and MT2-MMPs activate pro-MMP13. These activations form a cascade of events important in the degradation of ECM. MT1-MMP is widely expressed in normal tissues as well as in tumours. It can cleave a number of ECM proteins (Table 1.4). MT2-MMP is expressed in placenta, testis, colon, intestine, pancreas, kidney, lung, heart and skeletal muscle. MT3-MMP is found in brain, placenta, heart and certain malignant melanomas. MT4-MMP is expressed in brain, leukocytes, colon, ovary, testis

and breast carcinoma. MT5-MMP is predominantly expressed in kidney, pancreas, lung and brain tissues, especially brain tumours. Finally, MT6-MMP is expressed specifically by peripheral blood leukocytes and in lung and spleen tissue (Reunanen & Kähäri 2002).

Other MMPs include MMP-19 which is found in high amounts in acutely inflamed synovial cavities; MMP-28 may sometimes be found in basal keratinocytes at the wound edge; MMP-20, enamelysin has an expression pattern restricted to ameloblasts and odontoblasts of developing teeth. It is important in ECM remodelling during tooth development and was found to degrade amelogenin, aggrecan and cartilage oligometric matrix protein (Reunanen & Kähäri 2002).

1.6.1.2 Structure and activation of MMPs

MMPs have a multi-domain structure; all share common domain structures including a signal sequence, a propeptide, a catalytic domain containing the Zn-binding site, and a hemopexin-like domain (Stetler-Stevenson & Yu 2001). The signal peptide found at the N terminal of the molecule, directs the secretion of the proenzyme. The propeptide contains a highly conserved sequence in which the cysteine forms a covalent bond with the zinc ion and a cysteine switch that maintains the proMMP in latent form. The catalytic domain is made of two modules separated by a deep active site cleft with zinc ion at the bottom. The binding of the catalytic zinc is coordinated by three

ENZYME	MMP	SUBSTRATE
Soluble types:		
Collagenases:		
• ***Interstitial collagenases (collagenases-1)***	**MMP-1**	**Collagens I, II, III, VII, VIII, X and XI, gelatin, entactin, tenascin, aggrecan, FN, vitronectin, casein.**
• ***Neutrophil collagenases (collagenases-2)***	**MMP-8**	**Collagens I, II and III, aggrecan.**
• ***Collagenase-3***	**MMP-13**	**Collagens I, II, III, IV, IX, X, XIV, gelatin, FN, aggrecan, casein.**
Gelatinases:		
• *Gelatinase A*	MMP-2	Collagens I, II, III, IV, V, VII and X, gelatin, FN, laminin, aggrecan, elastin, vitronectin, tenascin.
• *Gelatinase B*	MMP-9	Collagens IV, V, XI, XIV, elastin, aggrecan, decorin, laminin, FN.
Stromelysins:		
• ***Stromelysin-1***	**MMP-3**	**Collagens III, IV, V, IX, X, and XI, gelatin, aggrecan, elastin, FN, vitronectin, laminin, entactin, tenascin, decorin.**
• ***Stromelysin-2***	**MMP-10**	**Collagens III, IV and V, gelatin, FN, elastin, aggrecan, casein, pro-MMP-1, pro-MMP-2, pro-MMP-9, pro-TNFα.**
Matrilysins:		
• *Matrilyisn-1*	MMP-7	Collagen IV, gelatin, aggrecan, link protein, elastin, FN, vitronectin, laminin, entactin, decorin, tenascin, casein, pro-MMP-1, pro-MMP-2, pro-MMP-9, pro-TNFα.
• *Matrilysin-2*	MMP-26	FN, fibrinogen, vitronectin, gelatin.
Others:		
• ***Stromelysin 3***	**MMP-11**	
• ***Metalloelastase***	**MMP-12**	**Ealstin, collagen IV, gelatin, FN, vitronectin, laminin, entactin, aggrecan.**
• ***No name***	**MMP-19**	**Collagen type IV, laminin, FN, gelatin, aggrecan.**
• ***Enamelysin***	**MMP-20**	**Amerogenin**
• ***CA-MMP***	**MMP-23**	**Gelatin**
• ***No name***	**MMP-27**	**Not known**

• *Epilysin*	MMP-28	Casein
Membrane types:		
Transmembrane:		
• *MT1-MMP*	MMP-14	ProMMP-2, proMMP-13, collagens I, II and III, gelatin, FN, vitronectin, laminin-1 and -5.
• *MT2-MMP*	MMP-15	proMMP-2, laminin, FN, tenascin, entactin, aggrecan, perlecan.
• *MT3-MMP*	MMP-16	proMMP-2, collagen III, FN.
• *MT5-MMP*	MMP-24	proMMP-2, gelatin
GPI-anchored:		
• *MT4-MMP*	MMP-17	Fibrinogen, fibrin, proTNFα.
• *MT6-MMP*	MMP-25	Collagen IV, gelatin, fibrinogen, FN.

Table 1.4 Mammalian MMPs (Itoh & Nagase 2002).

histidine residues and is essential for the proteolytic activity of the MMP (Reunanen & Kähäri 2002). At the C terminus, a proline-rich pivot region links the catalytic domain with the C-terminal hemopexin domain. This domain is a highly conserved one containing four repeats showing similarity to hemopexin plasma protein. The ends of the domain are connected by a disulfide bridge (Figure 1.9 (A)) (Reunanen & Kähäri 2002). MMP-2 and MMP-9 have, in addition, a gelatin-binding domain that contains three FN-type II repeats (Stetler-Stevenson & Yu 2001).

Membrane-type MMPs are further subdivided into transmembrane type, which contain a transmembrane domain and a short cytoplasmic tail at the C-terminus, and glycosylphosphatidylinositol (GPI)-anchored type which have a hydrophobic GPI-anchoring signal sequence at the C-terminus (Itoh & Nagase 2002). MT-MMPs contain a carboxy-terminal transmembrane domain after the hemopexin domain and reside on the cell surface (Stetler-Stevenson & Yu 2001) (Figure 1.9 (B)).

All MMPs are synthesized as inactive zymogens, with the exception of stromelysin-3 (MMP-11) and MT-MMPs, and are activated by a variety of proteases including plasmin, uPA and other MMPs (Nagase & Woessner 1999; Stetler-Stevenson & Yu 2001; Reunanen & Kähäri 2002). Furthermore, with the exception of MMP-11, -23 and-28, all soluble MMPs are activated extracellularly by proteinases (Itoh & Nagase 2002). MMPs -11, -23 and -28 are

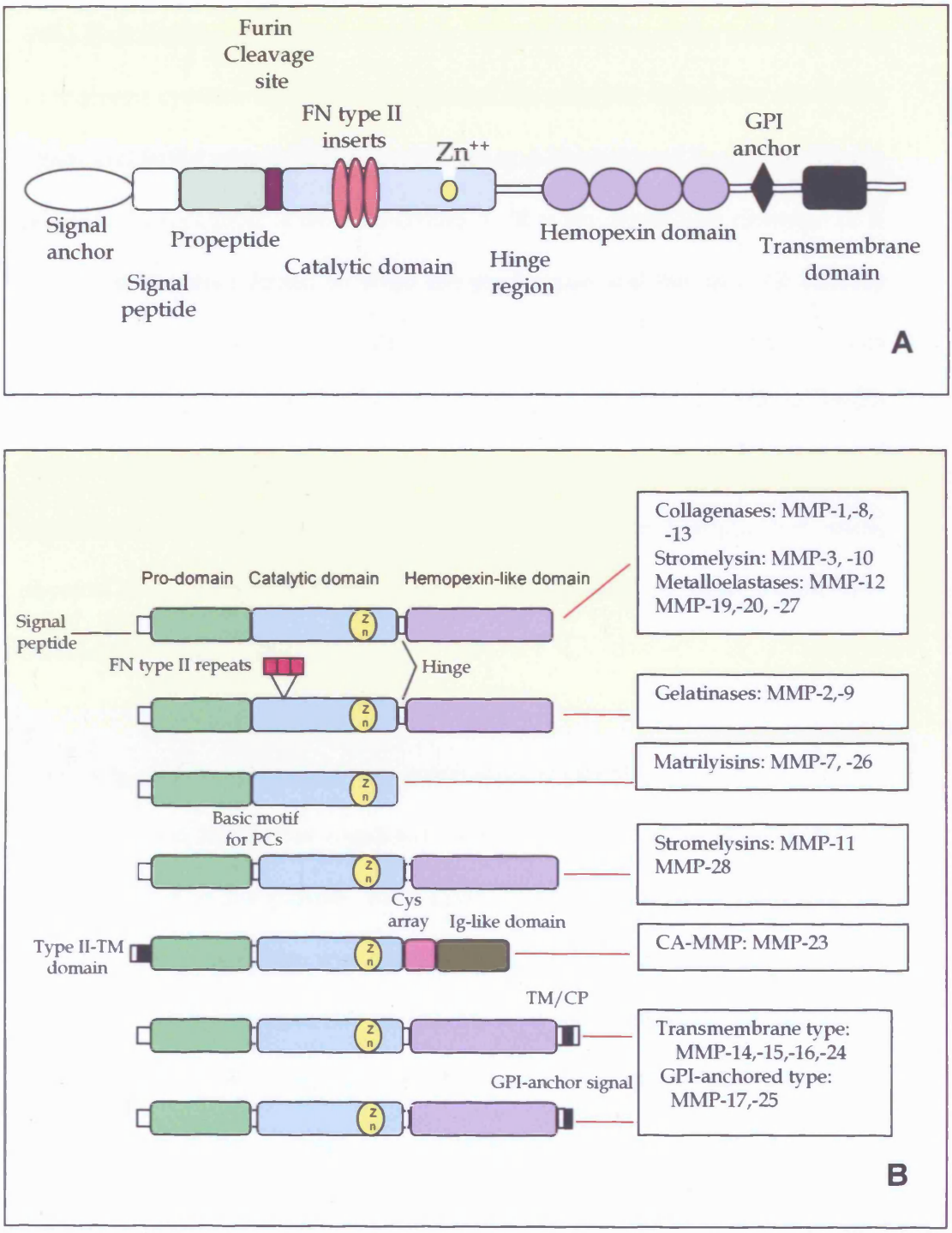


Figure 1.9 Conserved structure of MMPs (A) and doman structure of MMPs (B). PC: pro-hormone convertase, cys arrays: cys array domain,TM/CP: transmembrane and cytoplasmic domain (taken after Reunanen & Kähäri 2002; Itoh & Nagase 2002).

activated intracellularly and secreted as active molecules (Itoh & Nagase 2002). Extracellular activation occurs by the modification of the bond between a conserved cysteine sulfhydryl group and the catalytic zinc in the active site which yields the release of the prodomain and liberation of the active site for catalysis. Intracellular activation occurs by a furin-dependent cleavage of a conserved sequence found between the prodomain and the catalytic domain (Stetler-Stevenson & Yu 2001). MMP activity is controlled by anchoring them to the cell surface therefore localizing their activity to the pericellular space (Reunanen & Kähäri 2002). At the transcription level MMPs expression is regulated by growth factors, cytokines, chemical agents like phorbol esters, physical stess, oncogenic transformation, cell-cell and cell-ECM interactions (Reunanen & Kähäri 2002).

1.6.1.3 Tissue inhibitors of metalloproteinases (TIMPs)

Once activated MMPs are regulated by either TIMPs in the ECM or by α_2-macroglobulin in the plasma. Four TIMPs have been described, all ranging between 21-29kDa in size and consisting of conserved cysteine residues that form 6 intramolecular disulfide bonds. Three of these bonds are located in the N-terminal domain which also contains the MMP-inhibitory site and the C-terminal domain is involved in other binding interactions (Stetler-Stevenson & Yu 2001).

TIMPs have other biological functions distinct from the inactivation of MMPs (Nagase & Woessner 1999). TIMP-1 and TIMP-2 both exhibit mitogenic activities on various cell types and their over-expression inhibits tumour cell growth. TIMP-2 inhibits basic fibroblast growth factor-induced human endothelial cell growth (Nagase & Woessner 1999). TIMP-1 seems to stimulate the production of MMP-1 and may participate in cell growth. TIMP-3, by stabilizing TNF-α receptors induces apoptosis of human colon carcinoma cell lines (Nagase & Woessner 1999).

1.6.1.4 Functions of MMPs in pathologic conditions and tumours

As previously mentioned, MMPs are expressed in both physiologic and pathologic conditions. Collagenases are highly expressed during chronic ulcers, rheumatoid arthritis, gingival wounds, periodontitis and osteoarthritis (Reunanen & Kähäri 2002). Stromelysins are found during chronic wound healing and stromelysin-1 deficient mice showed impaired dermal wound contraction (Bullard *et al.* 1999). Gelatinases play a vital role in wound healing as they degrade the basement membrane to enable cells, such as T lymphocytes to migrate through to various sites. Deletion of MMP-2 in mice leads to impaired macrophage recruitment (Hautamaki *et al.* 1997). MT1-MMP deficient mice show severe defects in skeletal development and angiogenesis and activation of pro-MMP-2 is deficient (Reunanen & Kähäri 2002).

Matrix metalloproteinases also play a critical role in tumour growth and metastasis. By degrading the ECM they promote tumour growth and spread and play a role in enhancing angiogenesis (Reunanen & Kähäri). Collagenases, stromelysins, gelatinases and MT-MMPs have all been implicated to play important roles in tumourigenesis. MMP-11 deficient mice showed reduced chemical-induced tumourigenesis suggesting a role for this MMP in cancer cell survival (Masson *et al.* 1998; Bullard *et al.* 1999). Gelatinase-A and -B deficient mice show reduced angiogenesis, tumour progression, impaired metastasis formation and tumour growth (Itoh *et al.* 1998; Itoh & Nagase 2002). Many studies have established a positive correlation between MMP expression and the invasive and metastatic potential of tumours including colon, lung, head and neck, basal cell, breast, thyroid, prostate, ovarian and gastric carcinomas (Westermarck & Kahari 1999).

1.6.1.5 MMP inhibitors in clinical trials

Because of the significant role MMPs play in tumour progression, it would seem that targeting them could offer an effective therapy. Both endogenous MMP inhibitors (TIMPs) and synthetic inhibitors have been tested as potential treatment agents. Preclinical trials have been encouraging in that these inhibitors were able to block tumour cell invasion in vitro and in vivo (Brown 1997). Clinical trials have not been as successful on account of the lack of good bioavailability and the broad spectrum of these inhibitors (Brown 1998;

Nelson *et al.* 2000). Ideally, selective inhibitors aiming at specific MMPs are desirable. However, encouragingly it was found that they can nonetheless be administered as adjuncts of conventional cytotoxic therapy (Brown 1997).

1.6.2 The plasminogen activator system

The PA system is a cascade of serine proteases and which is initiated by plasminogen activators, of which there are two types, urokinase type (uPA) and tissue type (tPA). These act on the proenzyme plasminogen to form active plasmin. Plasmin is able to breakdown a number of molecules such as FN, laminin, vitronectin, proteoglycans and fibrin and can activate latent collagenases (Stoppelli 2005). This activity is controlled by the action of plasminogen activator inhibitors (PAIs) and alpha-2-antiplasmin (Preissner *et al.* 2000) (Figure 1.10). This system is not only important in fibrin dissolution and the maintenance of vascular patency but also in normal tissue remodelling, ECM degradation, and cell migration (Stoppelli 2005). Studies have shown that lack of any of its components can severely delay or impair wound healing and development (Vassalli & Pepper 1994; Ellis 1997; Edwards & Murphy 1998). UPA is relatively widely expressed by a variety of tissues and cells including proximal and distal kidney tubules, sub-epithelial fibroblasts in the gastrointestinal tract, bladder urothelium, ovarian granulosa cells and blood plasma. In pathologic situations such as invasive cancer, wound healing and activated leukocytes uPA is upregulated (Ellis & Dano 1991). TPA expressed by endothelium but very few other cell types. Vascular

smooth muscle cells express significant levels following vascular injury (Ellis 1997). It is now well established that elevated levels of uPA, PAI-1 and uPAR correlate with poor prognosis in tumours (Andreasen *et al.* 1997). This system plays an important role in tissue regeneration, wound healing, immune responses, angiogenesis and cancer invasion (Stoppelli 2005).

1.6.2.1. uPA

uPA was first discovered in human urine, hence the term urokinase. The interaction with its specific cellular receptor urokinase plasminogen activator receptor (uPAR) leads to plasma membrane localization and catalytic amplification (Vassalli & Pepper 1994; Ellis 1997). uPA is a serine protease that consists of two disulfide bridge-linked polypeptide chains. Therein are contained three functionally independent domains: a)- the serine proteinase domain, b)- the amino-terminal growth factor-like domain (containing the uPAR binding site) and c)- the single kringle domain (Andreasen *et al.* 1997; Reuning *et al.* 1998b) (Figure 1.10). The inactive precursor of uPA secreted by the cells as pro-uPA is a one-chain zymogen form with several-hundred-fold lower activity than the two-chain active uPA (Andreasen *et al.* 1997). The activation of pro-uPA to uPA is catalysed by several proteinases including plasmin, plasma kallikrein, blood coagulation factor XIIa, cathepsin B, cathepsin L and nerve growth factor-γ (Reuning *et al.* 1998a). uPA has a restricted substate specificity and plasminogen is the main substrate. Other molecules with sequences similar to plasminogen which may be substrates for

uPA include hepatocyte growth factor (HGF), uPAR and macrophage-stimulating factor (MSF) (Vassalli & Pepper 1994; Andreasen *et al.* 1997; Ellis 1997; Preissner *et al.* 2000). uPA has been found to be able to degrade ECM molecules such as FN independent of plasmin activity, and to activate other ECM-degrading enzymes such as MMPs (Reuning *et al.* 1998a).

1.6.2.2 uPAR

Membrane receptors of the PA system mediate plasminogem activation at the cell surface, thus focusing proteolysis at the pericellular environment (Stoppelli 2005). The urokinase receptor binds uPA with high affinity and is found on the surface of many cells including tumour cells. Many studies have found a correlation between the expression of uPAR and the migratory and invasive potential of tumour cells (Preissner *et al.* 2000). uPAR is a glycolipid-anchored membrane protein consisting of three homologous independently folded domains (domain1,2 and 3) (Andreasen *et al.* 1997) (Figure 1.10). It is anchored to the plasma membrane by a GPI moiety and this renders it quite mobile on the plasma membrane (Preissner *et al.* 2000). Studies have shown that uPAR distribution on migratory cells is different from stationary cells in that uPAR expression is clustered at the leading edge (Reuning *et al.* 1998a). uPAR may also be found in solution and differs from the cell-bound uPAR by the absence of the GPI domain (Andreasen *et al.* 1997). uPAR binds to the N-terminal growth factor-like domain of uPA (Andreasen *et al.* 1997). Other ligands of uPAR include vitronectin (to which it binds with high affinity), β2

integrins and β1 integrins (Andreasen *et al.* 1997). Through such alternative interactions uPAR has been linked with chemotaxis, adhesion and intracellular signal transduction cascades (Preissner *et al.* 2000).

Both uPA and pro-uPA bind to uPAR. Upon binding, pro-uPA is more susceptible to activation by proteases including plasmin (Reuning *et al.* 1998a). Evidence suggests that intracellular signals are initiated by the binding of uPA to uPAR which is independent of plasmin generation (Andreasen *et al.* 1997).

1.6.2.3 tPA

In contrast with uPA, tPA is secreted proteolytically active. The major role for tPA is in vascular biology. However, the finding of a specific tPA receptor in neuronal cells has implicated it to play a role in neural plasticity (Ellis 1997). The stimulation of tPA activity is dependent on the simultaneous cellular binding of plasminogen (Ellis 1997).

1.6.2.4 Plasminogen activator inhibitors (PAI-1 and -2)

PAI-1 and PAI-2 belong to the serpin superfamily and inhibit the catalytic activity of both uPA and tPA (Andreasen *et al.*1997). PAI-1 is synthesized by endothelial cells in high concentrations and by most neoplastic cells. PAI2 is synthesized by macrophages (Stoppelli 2005). Both PAI-1 and -2 regulate the activity of uPA by forming a complex with it that renders it inactive. PAI-1

can also bind to uPAR-bound uPA and this ternary complex prompts its rapid internalization and is followed by the degradation of uPA and PAI-1 by lysosomes and the recycling of uPAR back to the cell surface (Reuning *et al.* 1998a).

1.6.2.5 Plasmin

Plasmin is a broad-spectrum protease which may break down the ECM directly or indirectly by activating various MMPs (Ellis & Dano 1991; Vassalli & Pepper 1994; Preissner *et al.* 2000). Plasmin consists of two disulfide bridge-linked polypeptide chains, the C terminal consisting of a serine protease domain responsible for the catalytic activity and the N terminal consisting of 5 so-called kringle domains (Andreasen *et al.* 1997). These domains are responsible for the interaction between plasmin, plasminogen and the cell surface (Figure 1.10). Plasminogen can be cleaved into plasmin by the catalytic action of uPA, tPA and certain bacterial proteins (Andreasen *et al.* 1997).

1.6.2.6 pFN as substrtate for uPA

Studies have shown limited digestion of pFN by uPA (Gold *et al.* 1989). This type of ECM degradation is independent of plasmin. uPA was found to cleave the dimeric form of pFN into its two monomers, 220kDa each. However, smaller molecular weight fragments were also observed.

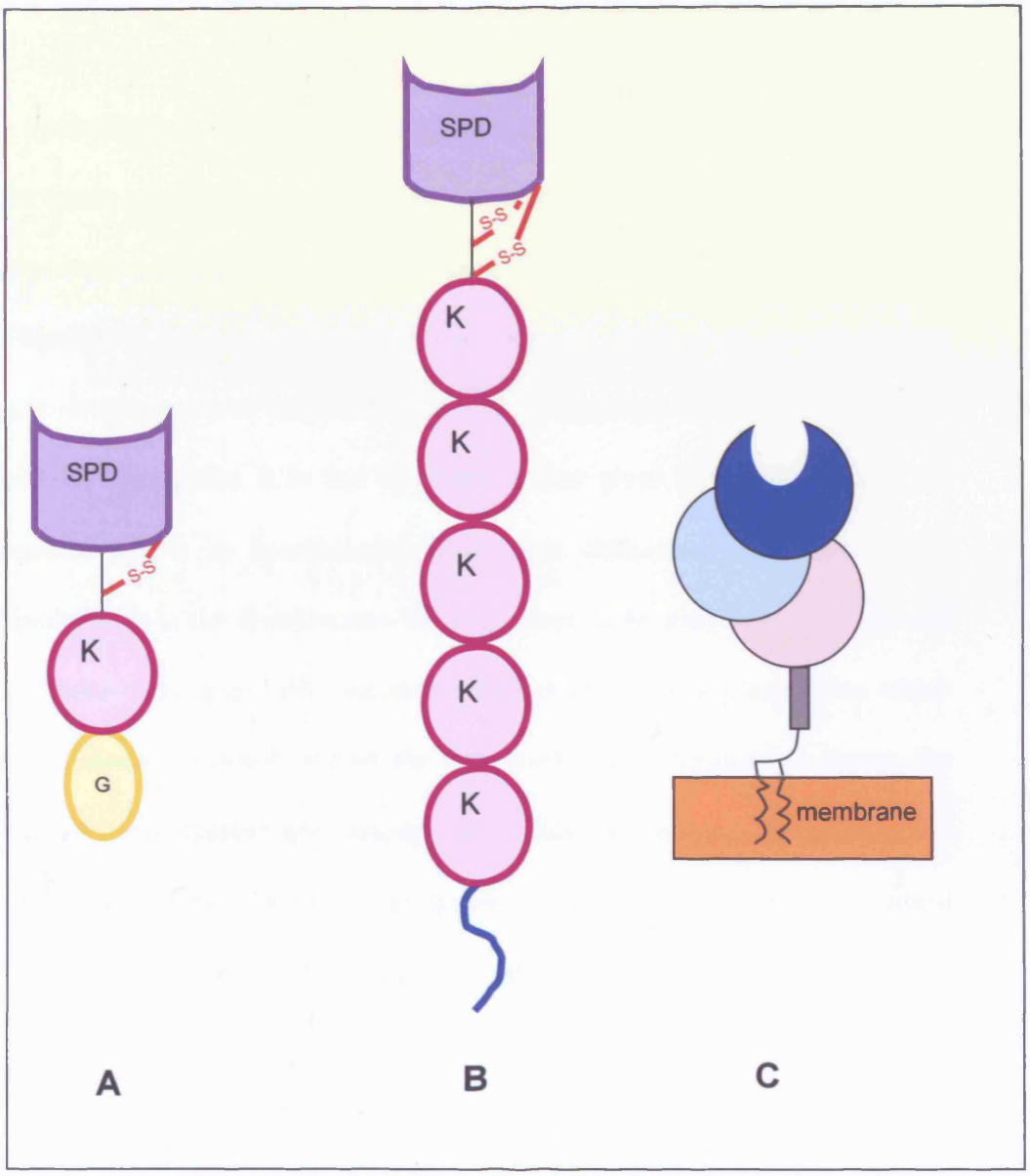


Figure 1.10 Domain structure of pro-uPA (A), plasminogen (B) and uPAR (C) where SPD is the serine protein domain, K is the Kringle domain, and G is the growth-factor domain (taken after Andearsen *et al.* 1997).

1.7 THE EPITHELIAL CELL

The epithelial cell is the basic component of the epithelium. Epithelial cells differ from other cells in the body in their content of tonofilaments and desomosomes (Ten Cate 1998). Tonofilaments are fibrous proteins that belong to a family of intermediate filaments known as cytokeratins and are important structural components within the cell. When aggregated they form bundles known as tonofibrils. It is this cytokeratin that gives the epithelial cell the alternative name of keratinocyte. The other characteristic component of epithelial cells is the desmosome. This structure is an area bordering the cell membranes of adjacent cells and consists of an attachment plaque into which the tonofilaments insert. When the cell borders the connective tissue, the specialized structures are known as hemidesmosomes. Together, the tonofilaments, desomosomes and hemidesmosomes form the mechanical linkage that distributes and dissipates forces applied on the cells (Ten Cate 1998).

There are two other junctions found apically between epithelial cells, the zonula adherens and the zonula occludens. The former is characterized by cadherin-mediated cell-cell adhesion and the latter by tight junction proteins such as occluding and the cytoplasmic plaque proteins ZO-1 and ZO-2 (Sander & Collard 1999; Evers *et al.* 2000). Tight junctions between cells and

the underlying basement membrane characterize epithelial tissues (Sander & Collard 1999).

1.7.1 The Cytoskeleton

The cytoskeleton is an elaborate network of protein filaments found throughout the cytoplasm. It is a highly dynamic structure that is continuously reorganized as the cell changes shape, divides and responds to outside stimuli (Bray *et al.* 1998). The cytoskeleton is responsible for movements such as crawling of cells along a surface, contraction of muscle cells and changes in cell shape. Furthermore, the cytoskeleton provides an intracellular channel system for transport and signalling. The main components of the cytoskeleton are the intermediate filaments, the microtubules and the actin filaments. The intermediate filaments are a type of fibrous proteins whose main function is to help the cell withstand mechanical stresses, for example when stretched. Microtubules and actin are made of tubulin and actin respectively. Microtubules are stiff hollow protein tubes that can be assembled and disassembled as required. They grow out of the centrosome to create the tracks that guide chromosomes during cell mitosis. They can also form permanent structures such as cilia and flagella (Bray *et al.* 1998).

1.7.2 Cell migration

Generally, epithelial cells are non-migratory. However, under certain circumstances they become motile and when this happens they undergo some form of mesenchymal transition by losing some epithelial characteristics such as polarity and cell-cell adhesion (Quaranta 2002). Cell migration can be both physiologic and pathologic. An example of physiologic migration is that of the basal cells in normal epithelium maturating and moving up towards the surface (Ten Cate 1998). Migration is also an integral part of both wound healing and cancer (Kassis *et al.* 2001). During wound healing epithelial cells migrate from the edges of the wound in a process called re-epithelialization (Hakkinen *et al.* 2000). Epithelial cells dissolve their hemidesmosomal connections and detach from the basement membrane in order to rapidly migrate over the wounded surface. An attribute of migrating cells is their ability to focus proteolysis at the leading edge of the epithelium (Hakkinen *et al.* 2000). Studies have shown the localization of urokinase type plasminogen activator receptor on the cell membrane at the advancing edge (Reuning *et al.* 1998a). Furthermore, other studies have shown increased expression of matrix metalloproteinase-9, matrix metalloproteinase-1 and matrix metalloproteinase-10 by migrating keratinocytes (Hakkinen *et al.* 2000). In carcinomas a similar process occurs. Malignant cells are known to attach to the basement membrane, secrete proteolytic enzymes and breach the barrier to penetrate the connective tissue beneath (Kassis *et al.* 2001) (Figure 1.8). These mutated migratory carcinoma cells also exhibit a very mesenchymal

phenotype and loose cadherin mediated cell-cell adhesion (Behrens 1999; Mercurio & Rabinovitz 2001; Wheelock & Johnson 2003). Needless to say this type of transition is associated with the most aggressive cancers (Mercurio & Rabinovitz 2001). The process of cell migration involves dynamic and coordinated interactions among ECM receptors, growth factor receptors and the actin cytoskeleton (Mercurio & Rabinovitz 2001). When growth factor receptors interact with the provisional ECM a cascade of events is triggered resulting in localized actin polymerization in the direction of cell movement. Traction forces are also established that help propel the cell forward (Mercurio & Rabinovitz 2001). Changes in the profile, localization and signalling of cell adhesion molecules such as integrins and cadherins is the hallmark of cancer progression (Evers *et al.* 2000; Kassis *et al.* 2001).

1.7.2.1 Epithelial cell migration modes

There are several morphogenetic movements that are relevant to epithelial migration and they include: sheet migration, tube migration, ingression, invagination and delamination. In order for these movements to occur there is the need for the downregulation of cell-cell adhesion and for the transition of epithelial cells to a more mesenchymal form (Quaranta 2002).

During *sheet migration*, the epithelia cover or envelop a tissue. It is a common mechanism during normal tissue development, wound healing and cancer invasion. When individual epithelial cells detach from the sheet and enter the

underlying stroma it is referred to as *ingression* (Quaranta 2002) and this is commonly seen during the migration of carcinoma cells through basement membranes. *Invagination* involves the formation of furrows, tubes or glandular structures by groups of epithelial cells within a flat epithelium and *tube migration* includes the formation and remodelling of glandular structures and secretory epithelia (Quaranta 2002) (Figure 1.11).

Such migratory mechanisms are often expressed by epithelial cells during normal physiologic processes such as embryonic development and tissue remodelling, or during pathologies such as wound repair and carcinogenesis (Quaranta 2002).

1.7.2.2 The dynamics of cell migration

Migration is facilitated by loss of cell-cell adhesion. This is achieved by loss in function and mutation of molecules mediating interactions such as E-cadherin and the catenins (Evers *et al.* 2000; Mercurio & Rabinovitz 2001). This is concurrent with the down regulation of hemidesmosomes despite the fact that in many cases the expression of the α6β4 integrin is maintained. However, this integrin is found in altered localizations such as lamellipodia (Kassis *et al.* 2001; Mercurio & Rabinovitz 2001). Such coordinated alterations are in many instances regulated by growth factors such as EGF, HGF and TGFα (Mercurio & Rabinovitz 2001).

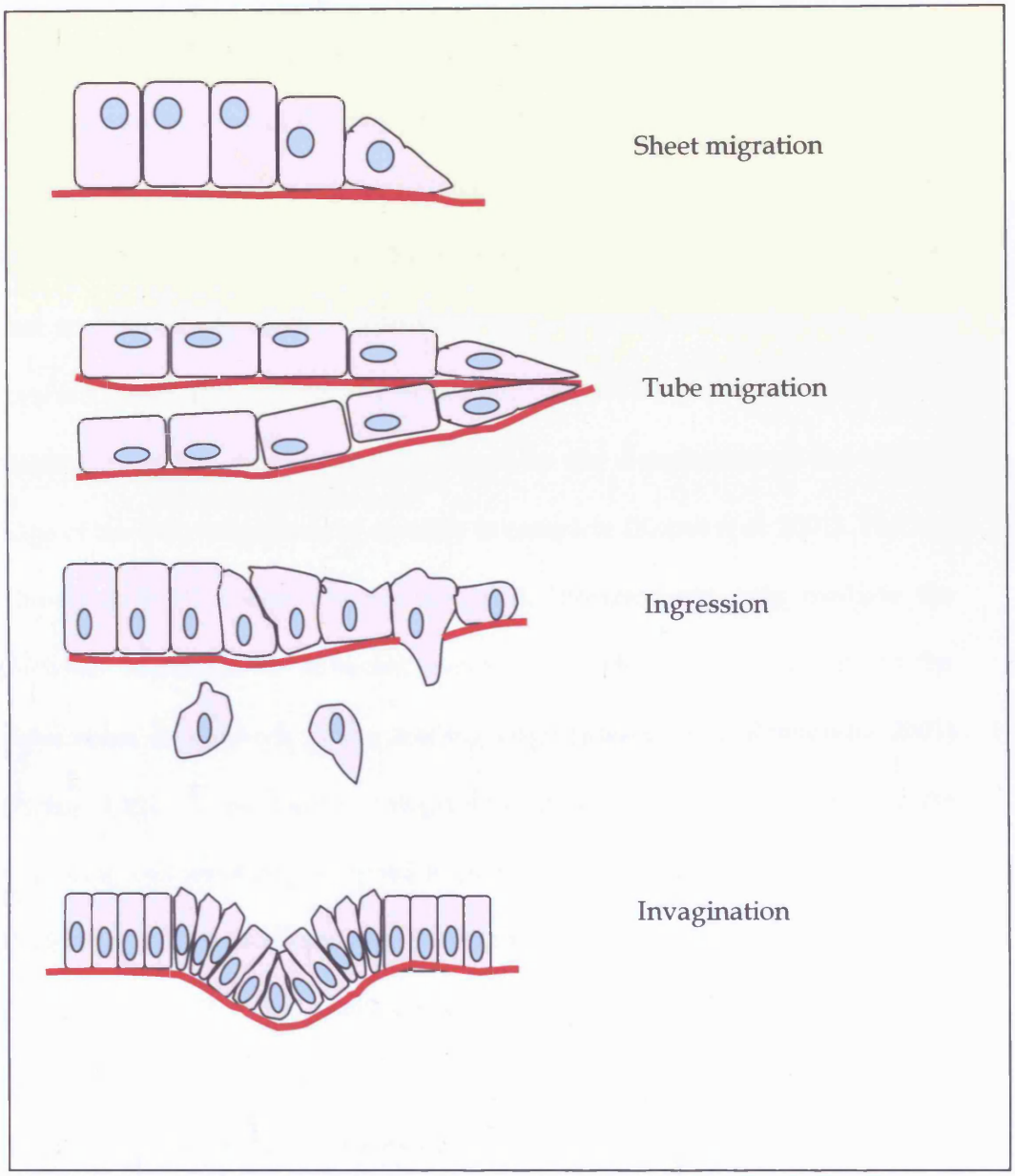


Figure 1.11 Schematic representation of epithelial movement (taken after Quaranta 2002).

The first step in cell motility involves the extension of the cell membrane via lamellipodia in any given direction (Kassis *et al.* 2001). If the lamellipodia is stabilized by adhesion molecules to the substratum, the actin polymerization generates protrusions at the edge of the cell which are followed by contraction of the actin network with associated myosin fibres (Kassis *et al.* 2001; Mercurio & Rabinovitz 2001). The actinmyosin system thus generates forces that are transmitted to the substrate creating the traction needed to produce forward movement resulting in the translocation of the cell body (Mercurio & Rabinovitz 2001). When this is followed by the detachment of the trailing edge of the cell, the process of motility is complete (Kassis *et al.* 2001). The key players in this process are the integrins. Integrins not only mediate the dynamic formation of adhesion contacts but also anchor the cell to the substratum and detach at the trailing edge (Mercurio & Rabinovitz 2001) (Figure 1.12). Concurrently, integrin-mediated adhesion at the rear of the migrating cell must be disrupted to permit forward movement (Mercurio & Rabinovitz 2001). Findings suggest that tumour cells shed integrins as they migrate through three-dimensional matrices, and this happens at the intracellular face of the cell membrane (Kassis *et al.* 2001). An important integrin involved in the active movement of cells is the $\alpha 6\beta 4$ integrin which is found to anchor filopodia to the ECM (Mercurio & Rabinovitz 2001). These stabilized filopodia then act as traction tracks for myosin and assist the formation of a spreading edge (Mercurio & Rabinovitz 2001). Similarly, the

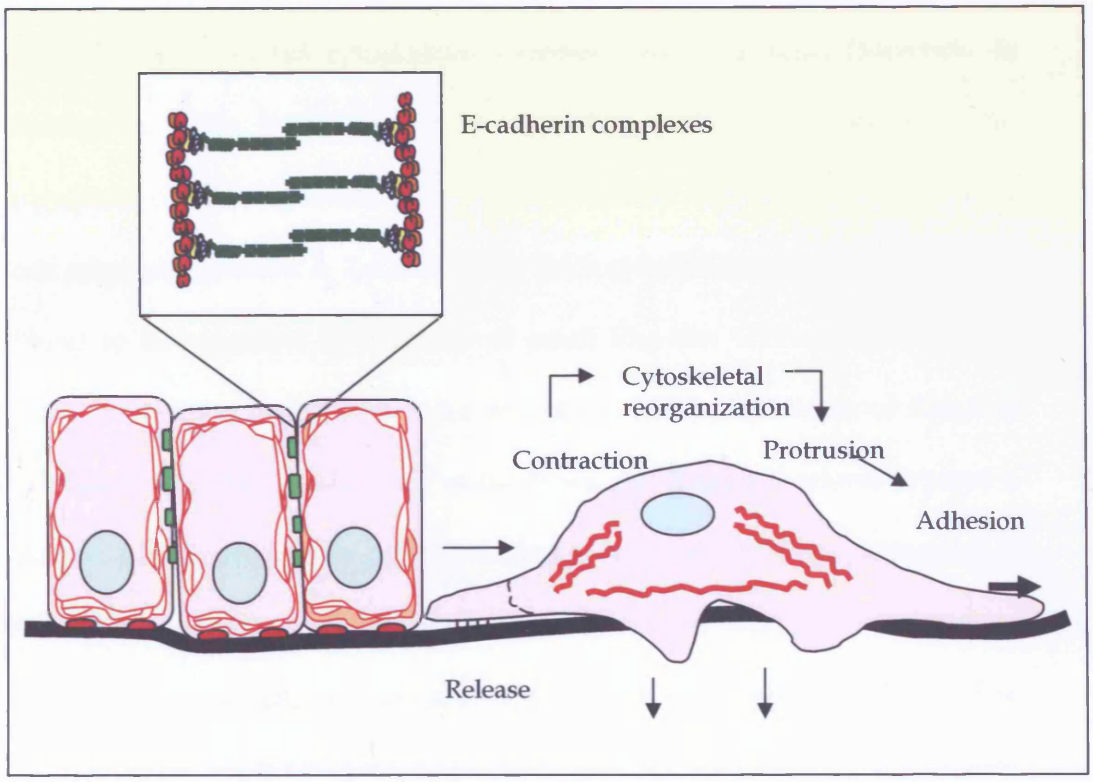


Figure 1.12 Dynamics of epithelial cell motility (taken after Evers *et al.* 2000 and Kassis *et al.* 2001). In preparation to movement cells downregulate their expression of E-cadherin complexes, relaxing the cell-cell adhesion. Cells extend protrusions in the form of lamellopodia and filipodia, adhesion is formed at the leading edge anchoring the cell to the stroma via cell-adhesion molecules such as integrins. This generates forces that cause cytoskeletal reorganization followed by contraction and the release of the trailing edge. In this way, bodily movement of the cell is achieved.

α6β4 integrin has been found localized at the base of lamellae in carcinoma cells migrating over ECM (Mercurio & Rabinovitz 2001).

1.7.2.3 Signalling in cell migration

Integrins work in synchrony with growth factors in regulating the signalling pathways that control cytoskeletal dynamics and migration (Mercurio & Rabinovitz 2001). Abnormalities in signalling pathways involved in the regulation of cell-migration, cell-cell and cell-ECM interactions contribute to cell migration (Sander & Collard 1999). Such cytoskeletal changes have been found to be regulated by a family of small Rho-like GTPases that include Cdc42Hs, Rac1 and RhoA (Sander & Collard 1999). Activation of RhoA is correlated with the assembly of stress fibres and focal adhesions; it plays a dual role with regards to cell-cell adhesion and motility. The activation of Cdc42Hs and Rac1 on the other hand is associated with the formation of lamellipodia and filopodia (Sander & Collard 1999; Evers *et al.* 2000). The epithelioid or migratory phenotype of the cells is determined by the balance of Rho and Rac activities. Cellular response to Rac1 activation, whether towards migration or not is determined in great part by signalling via integrin-ECM interactions (Sander & Collard 1999), and may be responsible for the loosening of intercellular adhesiveness due to functional disturbance of E-cadherin mediated cell-cell contacts (Evers *et al.* 2000).

Rac activation is controversial in that it may promote cell-cell adhesion and thus decrease migration or promote it. Its effect is dependent upon integrin signalling which is in turn dependent on cell-ECM interactions (Evers *et al.* 2000). Furthermore, there is cross-talk between Rac and Rho signalling; Rac activation leads to inactivation of Rho but the opposite is not true, suggesting unidirectional signalling (Evers *et al.* 2000).

1.8 SUMMARY:

The *de novo* expression of the αvβ6 integrin is of particular importance in both wound healing and tumourigenesis. This integrin establishes novel interaction between activated keratinocytes, tumour cells and their environment. Fibronectin is found in abundance in tissues and can be broken down to smaller, active fragments. Such fragments elicit responses unlike the parent molecule and could induce a more malignant phenotype. The αvβ6 integrin binds to the RGD sequence present in fibronectin and its cell-binding fragment. The expression of this integrin may therefore produce altered responses when it interacts with specific fibronectin fragments.

1.9 GENERAL AIMS:

The aim of this thesis is to investigate the interaction between various FN fragments and αvβ6-positive cells. Our choice of cell lines reflects our interest in the αvβ6 integrin, a *de novo* expressed integrin present in activated keratinocytes and tumour cells. The VB6 cells are a cell line transfected with the β6 integrin while the C1 are the null transfectant. Both cell lines are in essence the same except in their β6-integrin expression (Thomas *et al.* 1997). This particular integrin is known to bind to FN via the RGD sequence contained in the fibronectin repeat III_{10}. This makes the comparison between the intact molecule and the 120kDa FN cell-binding fragment interesting as both contain this sequence. The RGD sequence is known to bind to both the α5β1 and αvβ6 integrins and both these integrins are involved in cell migration. We aim to assess if this fragment evokes different effects on the two cell lines.

The β6 positive cells have been shown to produce more proteases than negative counterparts (Agrez *et al.* 1999). Our work will look at the secretion of the serine protease uPA and matrix metalloproteinases MMP-2 and -9. Such proteases have already been studied in relation to both wound healing and neoplasia and their expression often correlates with a more aggressive phenotype (Schedin *et al.* 2000; Davidson *et al.* 1999; Andreasen *et al.* 1997). Protease secretion may therefore be an important factor influencing the behaviour of our cell lines. Furthermore, it is important to evaluate the ability

of such proteases in generating the cell-binding FN fragment from the intact molecule. This may prove crucial in the loop implicating β6 positive cells in the production of specific fragments that elicit a pro-migratory response. *In vivo* correlation of β6, proteases and substrate could provide further evidence to the hypothesis we are proposing.

In order for us to draw credible conclusions from our results the repertoire of the critical cell-adhesion molecules expressed by both cell lines has to be evaluated. This will enable us to associate possible effects elicited by the FN fragments to the expression of a particular integrin. The assessment of high β6-integrin expression by the VB6 cells is crucial to our work. Furthermore, the establishment of the optimal medium of growth for our experiments is equally important. Most keratinocyte growth media contain fibronectin that may obscure the effects of the matrix proteins added by us in the various assays. In order to draw plausible conclusions, the effect of such "extrinsic" fibronectin must be kept to a minimum while growing the cells in a medium that does not jeopardize their growth or cell-adhesion molecule expression.

CHAPTER 2

MATERIALS AND METHODS

2.1 CELL CULTURE

The cell lines used in this study are the VB6 and C1 as tumour cells, and the UPs and normal human keratinocytes (NHK). VB6 and C1 are derived from a parental squamous cell carcinoma cell line, H357 (Prime *et al.* 1990) which is αv-negative (Figure 2.1). This was transfected with αv cDNA to create the αv-positive cell line, V3 (Jones *et al.* 1996) which express mainly αvβ5. The V3 cell line was further transfected with β6 to yield the αvβ6-positive cell line (VB6). C1 are the null transfectants of V3 (Thomas *et al.* 1997). NHK cells were provided by Cancer Research UK and were obtained from foreskin. UPs are normal keratinocytes immortalized for tissue culture by transfecting them with a viral oncogene, simian virus 40 (SV40) (Pei *et al.* 1991). Like NHKs, they express little αvβ6 and α5β1. All cells were grown and maintained in keratinocyte growth medium (KGM) in a humidified incubator at 37°C and 5% CO_2 (Appendix 1).

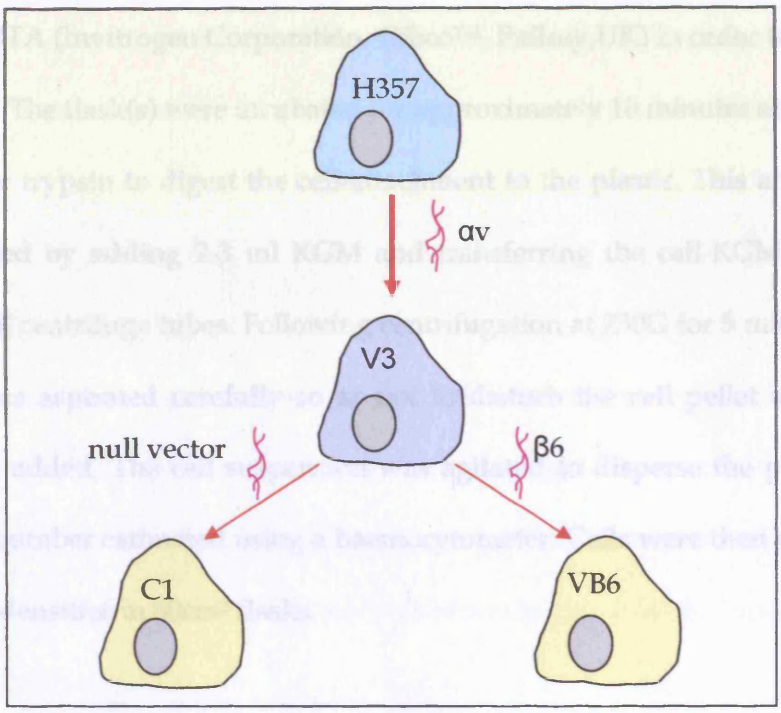


Figure 2.1 Diagrammatic representation of C1 and VB6 transfection.

2.1.1 Cell culture passage

All cell manipulations were carried out in a class II laminar flow cabinet to provide a sterile environment where growth medium was aspirated and the cell layer was washed twice with PBS containing 1mM calcium chloride and 1mM magnesium chloride. Cells were then covered with 1ml 0.05%Trypsin-0.02%EDTA (Invitrogen Corporation, Gibco™, Pailsey,UK) in order to harvest the cells. The flask(s) were incubated for approximately 10 minutes at 37°C to allow the trypsin to digest the cell-attachment to the plastic. This action was terminated by adding 2-3 ml KGM and transferring the cell-KGM mixture into 15ml centrifuge tubes. Following centrifugation at 230G for 5 minutes the KGM was aspirated carefully so as not to disturb the cell pellet and fresh medium added. The cell suspension was agitated to disperse the pellet and the cell number estimated using a haemocytometer. Cells were then seeded at varying densities in 80cm^2 flasks.

2.1.2 Counting the cells

During routine passage, after re-suspending the cells in known amounts of fresh medium and prior to seeding, 8µl of cell-containing medium was aspirated and placed under the coverslip of a haemocytometer. The cell containing medium fills the chamber by capillary action. The chamber is viewed under a phase-contrast microscope and the cells counted. The concentration is equal to the number in each 4 x 4 grid x10^4 /ml.

2.1.3 Freezing cell stocks

In order to maintain a supply of cell stocks, the cells were routinely frozen and stored in liquid nitrogen. During routine passage, after centrifugation the cells were counted and re-suspended in cold KGM containing 10% dimethylsulphoxide (DMSO), (Sigma-Aldrich Co Ltd., Dorset, UK). They were then transferred in cryovials at 1 million cells per 1ml medium and stored overnight at -80°C. The following day they were transferred to liquid nitrogen.

2.1.4 Thawing cell stocks

Cells were taken out of liquid nitrogen and the vials held in a 37°C water bath for rapid de-frosting. The cells were then transferred in a tube containing fresh medium, were centrifuged and the supernatant was then discarded. Cells were resuspended and seeded in flasks in approximately 14ml of KGM and incubated at 37°C.

2.1.5 Cell sorting

Rationale

This technique employs the use of magnetic beads designed for cell separation with any subclass of mouse IgG. Dynabeads bind to specific cell surface antigens via the primary mouse IgG used; target cells form rosettes with the Dynabeads Pan Mouse IgG (Figure 3.1) and these can then be

separated by applying a magnet (Dynal® Magnetic Particle Concentrator, Dynal Biotech) (Figure 2.2).

Procedure

Cells were grown to full confluency, trypsinized, resuspended in KGM and placed in 1.5ml microtubes (Sarstedt Ltd., Leicester, UK). The primary antibody, monoclonal mouse anti-β6 (R6G9, Chemicon International) was added and left for 30 minutes on ice. One hundred µl of Dynabeads suspension (Dynabeads® Pan Mouse IgG, Dynal Biotech) was added to 900µl KGM and washed twice by passing them along the magnet and discarding the medium. After incubation the cells were washed twice and spun at 690G for 3 minutes. Cells were resuspended in 300µl KGM and 20µl of Dynabeads solution was added to each tube. Cells were left on ice for 30 minutes with 5 minute interval shaking. After incubation the cells were washed six times as described above, resuspended in KGM and seeded in 6-well plates (Nunc™ VWR International). Once confluent the cells were transferred to cell culture flasks.

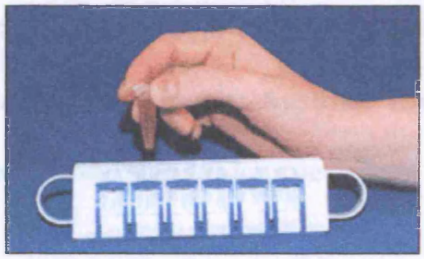


Figure 2.2 Dynal® Magnetic Particle Concentrator.

2.1.6 Proliferation assays

Proliferation assays were carried out using CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega UK, Southampton, UK) a colorimetric assay that indirectly measures viable cells. The MTS tetrazolium compound in the assay is bioreduced by dehydrogenase enzymes in live cells to give a coloured formazan bi-product soluble in tissue culture medium. The colour intensity in the medium is then measured at 490nm absorbance and plotted against a standard curve.

Using 96-well plates (Nunc™, VWR International, Dorset, UK) previously coated for 1 hour with matrix protein at 37°C, cells were seeded at a density of $30X10^4$ in triplicate and allowed to attach for 1 hour at 37°C. Cells were washed with PBS containing 1mM calcium chloride and 1mM magnesium chloride, 100µl SFM was added to each well and the plate returned to the incubator for a further hour. Twenty µl of MTS solution (Cell Titer 96® Aqueous One Solution Cell proliferation Assay, Promega UK) was added to the medium and the plates were incubated at 37°C for 1 hour in the dark. Following incubation the absorbance at 490nm was determined using a Plate Reader 340 ATTC (SLT Labinstruments, Austria). The number of cells was calculated by comparison to the standard curve.

2.1.7 Flow Cytometry

Flow cytometry is a method used to measure certain physical and chemical characteristics of cells or particles as they travel in suspension one by one past a sensing point. A modern flow cytometer consists of a light source, collection optics and a computer to translate signals into data (Figure 2.3). In the majority of cytometers the light source of choice is a laser which emits coherent light at a specified wavelength. The scattered and emitted fluorescent light is collected by two lenses and by a series of optics enabling

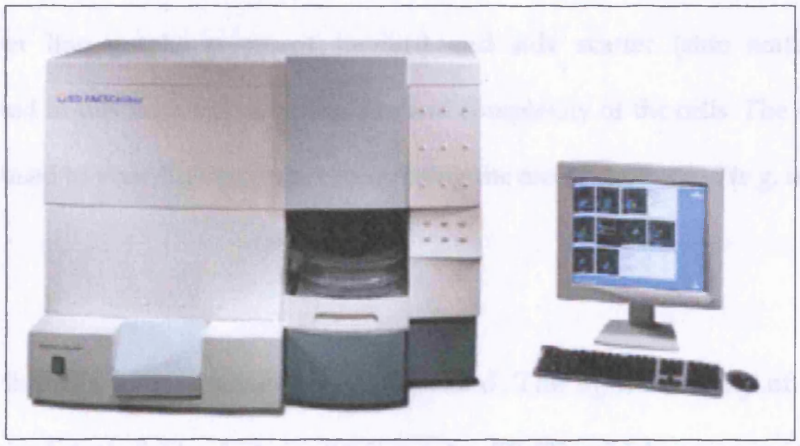


Figure 2.3 A Flow Cytometer connected to a computer for data analysis (http://wings.buffalo.edu/smbs/confocal/flow_cytometry.htm).

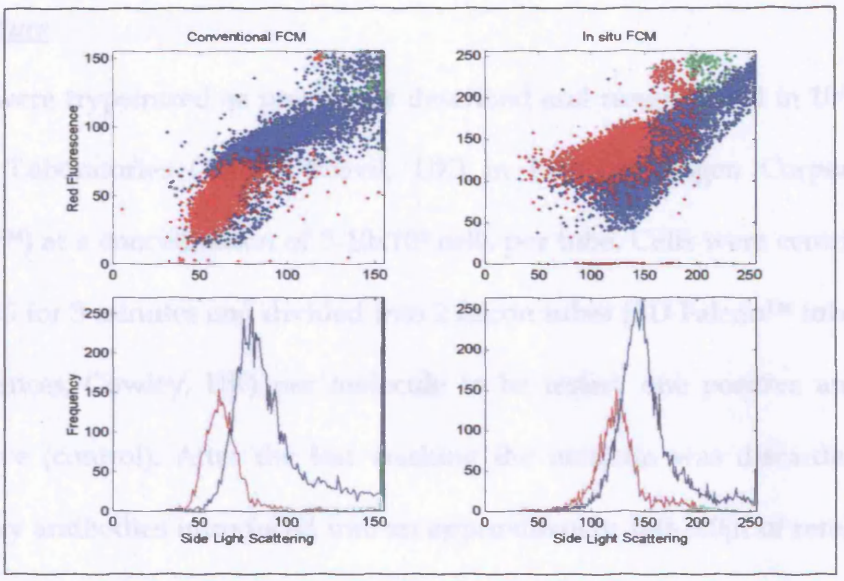


Figure 2.4 Graphic representation of Flow Cytometry data. The first two graphs are dot plots representing cell populations and the relative homogeneity of the sample. The lower line graphs represent forward and side scatter (side scatter being represented in this instance) denoting size and complexity of the cells. The same line graph is used to view fluorescence, representing the molecule studied (e.g. integrins).

specific bands of fluorescence to be measured. The light intensity of a cell or particle is detected by photomultiplier tube (PMT) which converts it via an amplifier to a voltage. Such data can be displayed using either a linear or a logarithmic scale (Figure 2.4). This method permits us to measure physical characteristics such as cell size, shape and internal complexity as well as any cell component (Vallan & Wider 2004).

Procedure

Cells were trypsinized as previously described and resuspended in 10% FCS (PAA Laboratories GmbH, Yeovil, UK) in PBS (Invitrogen Corporation, Gibco™) at a concentration of 5-10x10^5 cells per tube. Cells were centrifuged at 230G for 3 minutes and divided into 2 falcon tubes (BD Falcon™ tubes, BD Biosciences, Cowley, UK) per molecule to be tested, one positive and one negative (control). After the last washing the medium was discarded and primary antibodies introduced into an approximately 100-200µl of remaining medium and cells. The cells were left on ice for 1 hour after which medium (10%FCS in PBS) was added and they were spun again at 230G for 3 minutes. This washing step was repeated three times prior to adding the secondary antibody, rabbit anti-mouse FITC (Dako Cytomation, Ely, UK) at a concentration of 20µg/ml for 30-60 minutes on ice and in the dark. 10% FCS in PBS was added and the cells were washed three times before being taken to the flow cytometers for analysis. Analysis was carried out using FACscan (Becton Dickinson Immunocytometry Systems, Oxford, UK) which was in turn connected to an apple Macintosh computer fitted with Cellquest software set to aquire 10^4 events.

2.2 MOTILITY ASSAYS

2.2.1 Migration Assays

Haptotactic cell migration was measured using polycarbonate Transwell® inserts (Corning Costar, Poole, UK). The underside of these inserts was coated with the matrix protein to be tested diluted in PBS (Figure 2.5). The inserts were coated in 250µl of this solution for 60 minutes at 37°C. Inserts were then blocked with migration buffer consisting of DMEM and F12 (Appendix 1) in a 3:1 ratio with 0.5% BSA (Sigma-Aldrich Co Ltd.). Six hundred µl of this buffer was placed in new wells in a 24 well plate and 100µl of the same buffer in the top compartment to ensure the polycarbonate membrane was totally immersed. The inserts were incubated at 37°C for 1 hour.

Cells were trypsinized, counted and resuspended in migration buffer at 1million cells per 1ml medium. The migration buffer in the upper compartment of the inserts was then removed and replaced with 100µl of cell-containing medium to give a seeding density of 100,000 cells per insert. The inserts were incubated at 37°C for 4 hours. The inserts were then fixed in 10% formalin for 10 minutes followed by staining in 0.5% crystal violet in 10% ethanol for 10 minutes. They were rinsed briefly in water and the cells in the upper surface removed with a cotton bud. Membranes were allowed to dry overnight, cut and mounted on a slide with DPX. Cells were counted with phase-contrast microscopy and the number per high power field recorded.

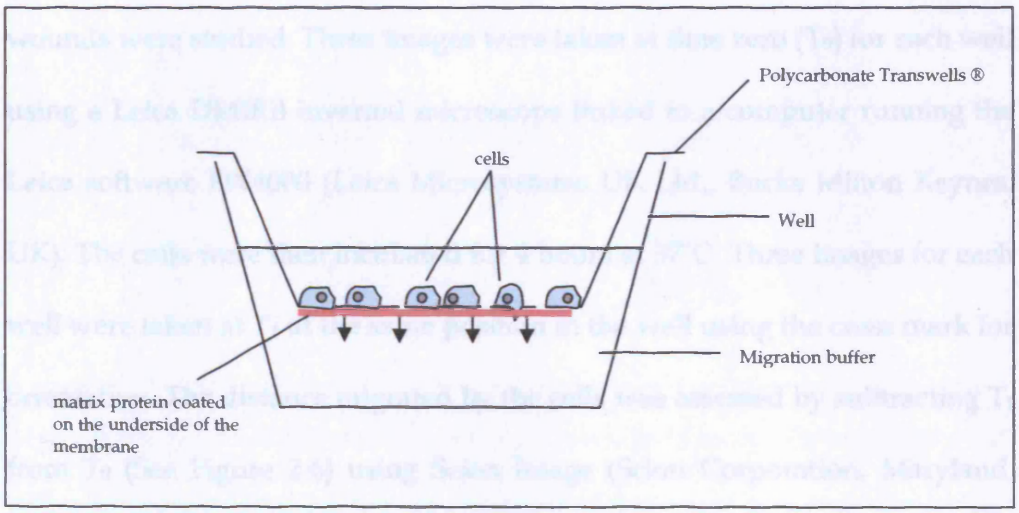


Figure 2.5 Haptotactic cell migration measured using polycarbonate Transwells ®. The underside Transwell® is coated with the matrix protein, cells are seeded in the upper compartment and are allowed to migrate over a period of time.

2.2.2 Wound Assays

Cells were grown to just below full confluence, trypsinized and seeded at 10^5 in 24 well plates. The cells were left overnight to reach maximal confluence then two denuded strips ('wounds') were created in each well with a pipette tip (P1000, Anachem Ltd., Bedfordshire, UK) in the form of a cross. Cells were once again washed with PBS to remove loosened cells. Care was taken to ensure the width of the wound was as constant as possible and only "similar" wounds were studied. Three images were taken at time zero (T_0) for each well using a Leica DMIRB inverted microscope linked to a computer running the Leica software FW4000 (Leica Microsystems UK Ltd., Bucks Milton Keynes, UK). The cells were then incubated for 4 hours at 37°C. Three images for each well were taken at T_1 at the same position in the well using the cross mark for orientation. The distance migrated by the cells was assessed by subtracting T_1 from T_0 (See Figure 2.6) using Scion Image (Scion Corporation, Maryland, USA).

2.2.3 Adhesion Assays

Using a 96 well plate triplicate wells were coated with 100µl of various matrix proteins to give a final dilution of 10µg/ml, for one hour at 37°C. The plate was washed once with PBS and blocked with 0.5% BSA in PBS for one hour at 37°C. Cells were washed in PBS, trypsinized and resuspended in serum-free medium (SFM). 30,000 cells were plated per well and left for one hour at 37°C.

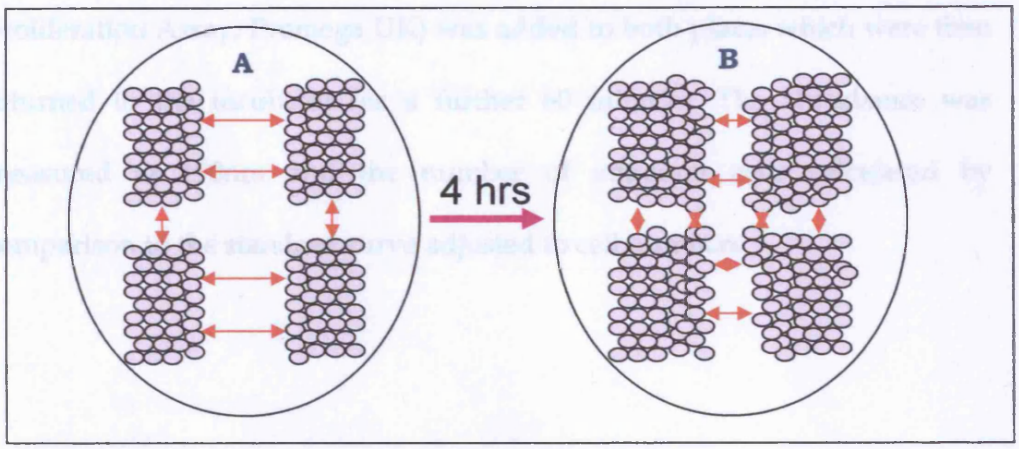


Figure 2.6 Schematic representation of a high power field image of a wound in a well plate. At T_0 (A) the distance between the edges of the wound is measured at various points and a mean is obtained. The same procedure is then carried out at T_1 (B).

This plate was then set aside. The plate with the samples to be tested was then washed in PBS containing 1.3mM $CaCl_2$ and 0.5mM $MgCl_2$ twice. 100µl of SFM was added to the wells and the plate was returned to the incubator for one hour.

At this point 20µl of MTS (CellTiter 96® Aqueous One Solution Cell proliferation Assay, Promega UK) was added to both plates which were then returned to the incubator for a further 60 minutes. The absorbance was measured at 490nm and the number of adherent cells calculated by comparison to the standard curve adjusted to cell numbers.

2.3 PROTEASE SECRETION ASSAYS

2.3.1 Plasminogen Activator Assay

This assay measures the concentration of PA in the conditioned medium of cells using a chromogenic substrate. The PA cleaves the plasminogen present in the reaction mixture to form the active enzyme plasmin. This reacts with the chromogenic substrate to release a colorimetric molecule (pNA) whose concentration can be measured at 405nm (Figure 2.7).

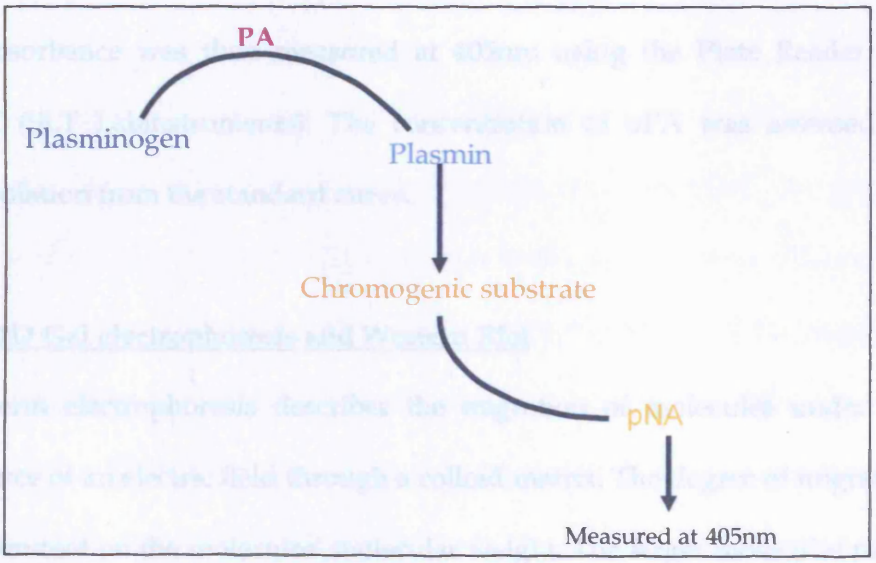


Figure 2.7 Diagrammatic representation of plasminogen activator assay.

Procedure

Cells were trypsinized as previously described and seeded in 6- or 24-well plates (Nunc™, VWR International) for 4 hours in KGM to enable the cells to attach, then after washing with PBS the medium was changed to SFM and the

cells were left overnight. The following day the supernatant was collected, cleared by centrifugation at 1150G for 5 minutes and kept on ice for analysis. Standard solutions were obtained by preparing a number of serial dilutions of uPA (Chemicon International) in 50mM Tris/100mM NaCl/0.01% Tween 80 (pH 7.4) and placed in 96-well plates (Nunc™, VWR International). Samples were also prepared in 0.01% Tris/Tween 80. Chromogenic substrate S-2251 (Chromogenix, Quadratech, Epson, UK) was added to all the wells at a final concentration of 2.5µg/ml and the reaction was activated by adding Glu-plasminogen (Enzyme Research Laboratories, Swansea, UK) at a final concentration of 160nM. The plate was incubated for 90 minutes at 37°C and the absorbance was then measured at 405nm using the Plate Reader 340 ATTC (SLT Labinstruments). The concentration of uPA was assessed by interpolation from the standard curve.

2.3.2 1D Gel electrophoresis and Western Blot

The term electrophoresis describes the migration of molecules under the influence of an electric field through a colloid matrix. The degree of migration is dependent on the molecules' molecular weight. The larger molecules move through the matrix with more difficulty whereas the smaller molecules are more dynamic. Resulting molecular bands show and depending on their location an approximate molecular weight may be deduced by use of known molecular markers.

Figure 2.8 Apparatus used during vertical electrophoresis (www.bio-rad.com)

Procedure

Protein Assay:

The Bio-Rad DC Protein Assay (Biorad, Hemel Hempstead, UK) was used according to the manufacturer's instructions, to determine the protein concentration in each sample. This assay is based on a colorimetric reaction between the protein in the sample and the copper tartrate and folin reagent in the buffer. The reaction yields a characteristic blue colour with maximal absorbance at 750nm. The protein level in cell supernatants was estimated by comparison to standard solutions of BSA. In this way samples containing the same amounts of protein can be subjected to electrophoresis.

Sodium Dodecyl Sulphate polyacrilamide gel electrophoresis (SDS-PAGE):

Supernatants containing 10µg total proteins were diluted in non-reducing sample buffer (Appendix 1) and subjected to SDS-PAGE electrophoresis using

a 10% acrylamide (Flowgen Bioscience Ltd., Nottingham, UK) resolving gel and 4% stacking gel (Appendix 1) in the appropriate apparatus (Figure 2.8). Molecular weight markers (Amersham Life Science, Little Chalfont, UK) were also loaded. Gels were run for 10 minutes at 100 volts, then 140 volts for 1 hour. After running the gel the assembly was dismantled and the gel carefully removed and stained using Commassie Blue stain (Appendix 1).

Membrane transfer:

Proteins were transferred to a nitrocellulose membrane (Opitban, Schleicher & Schuell, London, UK) overnight at 140V using a mini Trans-Blot cell assembly (Biorad). Once the transfer was complete the blot was incubated in blocking solution (5% dried milk in 0.05% tween20/PBS) for 1 hour with agitation. The blot was rinsed in PBS/0.05% Tween20 and incubated with primary antibody for 1 hour with agitation. The blot was rinsed three times in PBS/0.05% Tween20/0.1%BSA for 10 minutes each, incubated with secondary antibody polyclonal goat anti-mouse IgG, final conc. 0.3µg (Dako Ltd.) for 1 hour before repeating the three washing steps.

Development of the membrane

Specifically bound antibody was detected using enhanced chemiluminescence (ECL) according to the manufacturer's instructions (Amersham Life Science). In the dark room under safe light, 2ml of the ECL reagent mixture was pipetted over the blot and left for 1 minute. The blot was then wrapped in

cling film, placed in a cassette and exposed to radiographic film for varying lengths of time ranging from 15 seconds to 1 minute. The film was then immersed in developer followed by fixer and then washed in water and dried.

2.3.3 Zymography

Gelatin zymography is a technique that involves electrophoresis of secreted proteases (in this case, MMP-2 and MMP-9) through polyacrylamide gels containing the enzyme substrate gelatin. Following electrophoresis the SDS in the gel is removed by washing in 2.5%Triton-X (Appendix 1) to allow the enzyme to renature and degrade the protein substrate. The gel is then stained with commassie blue and the bands of proteolytic activity can be assessed as clear bands of lysis against a blue background.

Procedure

Gels containing 12% polyacrylamide and 10% gelatin (Sigma-Aldrich Company Ltd.) (Appendix 1) were prepared and equal volume samples (20µl) were loaded into the wells along with molecular weight standards (Kaleidoscope Prestained Standard, Bio Rad). The gels were run with 1X Tris-Glycine SDS Running Buffer (Appendix 1) at 120V for approximately 1 hour. The run is completed once the bromophenol blue tracking dye reaches the bottom of the gel. The gel is then washed twice in Renaturing Buffer (Appendix 1) for 30 minutes with gentle agitation at 37°C to remove

remaining SDS and then incubated in Zymogram Developing Buffer (Appendix 1) overnight again at 37°C. The following day the gels are stained with 0.5% Commassie Blue R-250 in 40% methanol, 10% acetic acid for up to 12 hours. The gel was then de-stained for 1 hour at room temperature until the clear lysis bands show. An image of the gel was taken by use of the Alpha imager 1220 version 5.5 (Alpha Innotech Corp™, USA). The images were then analysed using Scion Image (Scion Corporation).

2.4 IMMUNOHISTOCHEMISTRY

2.4.1 Indirect Immunofluorescence

Indirect immunofluorescence is a procedure carried out on cells or tissue sections whereby an antibody is used to link a cellular antigen specifically to a stain that can be more readily seen with a microscope. This procedure involves the detection of the antibody by fluorescence. A molecule known to fluoresce is attached to the antibody and is detected using UV light.

Procedure

Cells were seeded at a density of 10^4 overnight in Defined Keratinocyte serum-free medium (Invitrogen Corporation, Gibco™) on 13mm cover slips (Nunc™, VWR International Ltd.) placed inside 24-well plates (Nunc™, VWR International). The following day the cells were washed with PBS (Invitrogen Corporation, Gibco™) and fixed with 4% paraformaldehyde in PBS for 10 minutes at room temperature. After rinsing once with PBS they were treated with 0.1% TritonX-100 in PBS for 5 minutes at room temperature and were rinsed in PBS once more. Blocking was carried out with 0.1% sodium azide/0.1% BSA in PBS (0.1/0.1 solution) for 20 minutes at room temperature. Following the blocking step, the primary antibodies were added for one hour at 4°C (see chapter 6 for antibodies and concentrations). Negative controls were left without primary antibodies at this stage, in PBS to avoid the cells drying out. The cover slips were rinsed three times with 0.1/0.1 solution and the secondary antibody added, goat anti-mouse IgG FITC/Alexa-488

(Molecular Probes-Invitrogen Ltd., Paisley, UK) at a concentration of 20µg/ml for 30 minutes at room temperature in a dark environment. Cells were rinsed once and washed three times with 0.1/0.1 solution. A solution containing 4',6-Diamidino-2-phenyindole, dilactate (DAPI) and phalloidin-TRITC (Sigma-Aldrich Company Ltd.) at concentrations of 50µg/ml in 0.1/0.1 solution, was added for 10 minutes at room temperature and in the dark. After rinsing once and washing three times, each cover slip was dipped in distilled water (dH_2O), drained and mounted face down onto glass slides with anti-fade mountant, glycerol/PBS (Citifluor Ltd., London, UK). Slides were viewed using a Confocal Inverted Laser Scanning microscope (Zeiss Axiovert 200M, Welwyn Garden City, UK) and images collected digitally.

2.4.2 Immunohistochemistry

Coating of slides

A number of primary antibodies require some form of antigen retrieval for optimal binding to an antigen on a tissue section. Two methods can be used, heat and enzymatic retrieval. When using heat to retrieve the antigen, the slides used must be coated with APES (3-Aminopropyltriethoxysilane) to avoid the detachment of the section under the high pressure and heat. Slides are soaked in hot soapy water for about 10 minutes. They are rinsed in hot running water for another 10 minutes and then rinsed in 2 changes of deionised water for a few seconds each time. They are rinsed in Industrial

Methylated Spirit, IMS (Tennants Distribution Ltd., Manchester, UK) for a few seconds and dried in the oven at 40°C for 1 hour. Once dry the slides are dipped in 5% APES (Sigma-Aldrich Company Ltd.) in IMS for about 15-20 seconds, rinsed in IMS for a minute, rinsed in 2 changes of deionised water for 15-20 seconds each time and dried in the oven at 40°C overnight.

Antigen retrieval

In our work we carried out both types of antigen retrieval. Heat-mediated antigen retrieval was necessary for cFN staining whereas beta-6 integrin and cytokeratin required enzymatic retrieval by Pepsin (Zymed® Laboratories, Cambridge, UK) and Chymotrypsin (Sigma-Aldrich Company Ltd.) respectively for 5 minutes at room temperature.

Heat-mediated antigen retrieval:

In this procedure sections are cooked under pressure for 2 minutes in 2 litres of Citrate/HCL buffer (Appendix 1) at a PH of 6.0.

Procedure

Sections were dewaxed twice in xylene (Solmedia Lab Suppliers, Romford, UK) for 5 minutes each time. They were then rehydrated through decreasing concentrations of alcohol, 100%, 95% and 50% ethanol twice for 2 minutes each time. They were then immersed in distilled water for 2 minutes. Following antigen retrieval the sections were encircled with a PAP pen (Zymed® Laboratories). Endogenous peroxidase was then blocked using

peroxidase-blocking solution (ChemMate, Peroxidase-Blocking Solution, Dako Cytomation). Primary antibodies were applied for one hour at room temperature.

The sections were washed twice in Tris buffered saline (TBS), (Appendix 1) following which the secondary antibody was added (ChemMate™ DAKO Envision™/HRP, Rabbit/Mouse, Sigma-Aldrich), for one hour. Slides were again washed twice in TBS and diaminobenzidine tetrahydrochloride (DAB) added for 10 minutes. Specimens were washed with TBS followed by warm tap water and they were then stained with Mayer's haematoxylin (Appendix 1) for nuclear counterstain. They were washed once again with water and then rehydrated with ascending concentrations of alcohol, 50%, 95% and 100% ethanol followed by xylene, and they were finally mounted using Permount (Fisher Scientific, Loughborough, UK) and observed under the microscope.

2.5 STATISTICAL ANALYSIS

Data is expressed as the mean +/- standard deviation of a given number of values. Where needed, statistical analysis was carried out using non-parametric analysis of variance (ANOVA) to compare multiple groups. Comparisons were by Kruskal-Wallis Test between 3 groups or more, and the Mann-Whitney Test between 2 groups. Significance was set at 5% and a p value of <0.05 was considered significant.

CHAPTER 3

CHARACTERIZATION OF CELL LINES AND OPTIMIZATION OF CULTURE CONDITIONS

3.1 INTRODUCTION

Cells receive multiple cues from the environment they are in, which influences their phenotype and gene expression. Cell adhesion molecules such as integrins and cadherins mediate such environmental cues and are important determinants of cell morphology and behaviour. Cadherins are spatiotemporally regulated to correlate with specific morphogenetic and developmental events. It is not uncommon for tumour cells to downregulate their expression of E-cadherins as they change to a more mesenchymal phenotype. In fact, E-cadherin expression has been considered a prognostic marker of greater differentiation (Wheelock & Johnson 2003). There is established cross-talk between cadherins, integrins and their surrounding ECM that leads to the activation or inactivation of signalling pathways.

When studying the effect of a particular molecule on cell behaviour it is vital to optimize the culture conditions. The extra cellular matrix is a delicate balance of many molecules, cations and pH, and minor changes can influence responses in the cells. Baseline characterization was performed to establish the expression of cell adhesion molecules in relation to different medium constituents. Data contained within the ECM is vital for differentiative

decisions and normal cellular homeostasis, while inappropriate changes in both its structure and information content lead to disease states such as neoplasia (Streuli 1999). In cell culture the cells are surrounded by medium that contains many of the molecules found in vivo. Although the composition of the media is formulated to mimic as much as possible the tissue fluid and ECM, these two entities are not necessarily the same. Establishment of the most appropriate medium is therefore of importance since variations can influence cell behaviour, such as migration and differentiation.

Migration of various cells is determined by variables such as their origin, adhesion receptor function and the environment they are in including the nature of the ECM (Murphy & Gavrilovic 1999). Loss of specific components of the ECM has been found to affect the differentiation of specific cell types (Bissell & Nelson 1999). When normal keratinocytes are placed in suspension they are stimulated to undergo terminal differentiation and this can be partially inhibited by the addition of certain ECM molecules or anti-β1 antibodies into the medium, inducing cell adhesion. This suggests that adhesion normally suppresses terminal differentiation (Levy *et al.* 2000). Furthermore, cues provided by molecules in the media can regulate the expression of cell adhesion molecules, either inducing or inhibiting them. There appears a dynamic competition between integrins and cadherins for polymerized actin microfilaments (Weaver *et al.* 1997) and certain cues in the ECM microenvironment in vivo, or medium in culture, influence the balance

one way or another. Determining cell adhesion molecule expression of the cells prior to experiments is necessary to establish the effects of the manipulation of medium constituents.

The αvβ6 integrin is expressed *de novo* and is an indicator of a more aggressive cell phenotype (Thomas *et al.* 2001a). E-cadherin expression on the other hand is lost in the more aggressive lesions (Christofori 2003). The VB6 cell line used in this work expresses high amounts of the αvβ6 integrin whereas the C1 being their null transfectant express minimal amounts (see section 2.1). Studies on VB6 have found them to be more migratory and produce more proteases than their C1 counterparts (Thomas *et al.* 2001b) particularly when seeded on ECM molecules such as FN.

3.2 OBJECTIVES

To confirm the cell adhesion profile of the cell lines to be used in studies described within this thesis and to assess whether the cell adhesion molecules expressed by the cells are influenced by specific media components. Furthermore, we aim to establish if any change observed by manipulating medium additives is reversible and to investigate which particular cell-adhesion molecule is affected.

3.3 MATERIALS AND METHODS

3.3.1 Fluorescence Activated Cell Sorter (FACS)

Cells were grown in 25cm^2 culture flasks (Nunc™, VWR International) overnight at 37°C in KGM. They were trypsinized and resuspended in 10% FCS in PBS and centrifuged for 3 minutes at 230G at a concentration of about 5-10x10^5 cells per tube. The supernatant was discarded and primary antibodies added:

- Monoclonal mouse anti-β6 (R6G9, Chemicon International)
- Monoclonal mouse anti-E-cadherin (Clone HECD-1, Abcam Ltd, Cambridge, UK)
- Monoclonal mouse anti-α5 (P1D6, Chemicon International)

at concentrations of 10μg/ml, for one hour on ice. Five hundred μl of 10% FCS in PBS was added and the tubes were washed twice before adding the secondary antibody, FITC-conjugated rabbit anti-mouse immunoglobulin (Dako Cytomation) at concentration of 20μg/ml for 30-60 minutes on ice and away from light. Cells were washed three times as described above and then subjected to FACS analysis (FACscan, Becton Dickinson Immunocytometry Systems). The FACscan was in turn connected to an apple Macintosh computer fitted with Cellquest software set to aquire 10^4 events.

3.3.2 Cell sorting by magnetic beads

Rationale

During the preliminary integrin-profiling of the cell lines, a mixed population of VB6 was observed (figure 3.2 A) indicating that some cells were expressing the β6-integrin while others were not. This is a common finding in transfected cell lines where some cells either loose the expression of the transfected molecule or cells that from the start failed to be transfected grow and divide and subsequently form a sub-population. Whatever the reason, seeing that the β6 integrin was pivotal to our work, the VB6 were sorted to isolate the β6 positive population from the β6 negative one (Figure 3.1). This ensured a homogeneous population of β-6 expressing cell lines (Figure 3.2 B). The VB6 cells were sorted using magnetic beads according to the manufacturer's instruction (Dynal Biotech®, Wirral, UK). Cells were trypsinized and sorted as described in section 2.1.5.

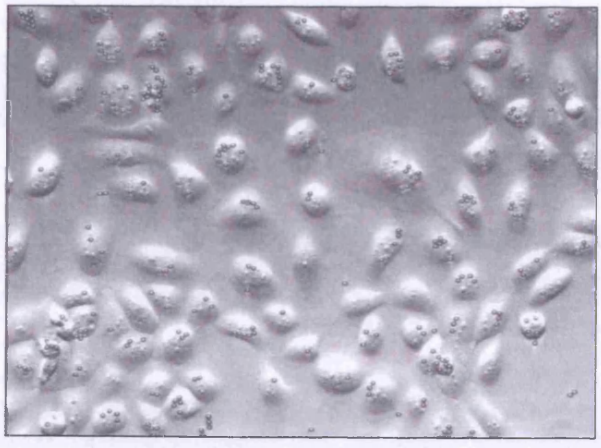


Figure 3.1 Sorted VB6 exhibiting the Dynabeads on their surface appeared as rosettes.

3.3.3 Growth of cells in various media

VB6 and C1 cell lines were grown in specified media (Table 3.1) for a duration of at least 5 days that included at least one passage, but averaged two. Media were made up, filtered and stored in sterile containers at 4°C.

MEDIUM	Constituents:
KGM	DMEM, F12, FCS, insulin, EGF, Penicillin/Streptomycin, adenine, cholera toxin, Fungizone, Hydrocortisone.
AFM	DMEM, F12, FCS, Penicillin/Streptomycin, Fungizone.
AFM-A	DMEM, F12, FCS, Penicillin/Streptomycin, Fungizone, insulin.
AFM-B	DMEM, F12, FCS, Penicillin/Streptomycin, Fungizone, EGF.
AFM-C	DMEM, F12, FCS, Penicillin/Streptomycin, Fungizone, cholera toxin.
AFM-D	DMEM, F12, FCS, Penicillin/Streptomycin, Fungizone, adenine.
AFM-E	DMEM, F12, FCS, Penicillin/Streptomycin, Fungizone, hydrocortisone.
SFM	DMEM, F12, Penicillin/streptomycin, Fungizone
SFM-A	DMEM, F12, Penicillin/streptomycin, Fungizone, insulin.
SFM-B	DMEM, F12, Penicillin/streptomycin, Fungizone, EGF.
SFM-C	DMEM, F12, Penicillin/streptomycin, Fungizone, cholera toxin.
SFM-D	DMEM, F12, Penicillin/streptomycin, Fungizone, adenine.
SFM-E	DMEM, F12, Penicillin/streptomycin, Fungizone, hydrocortisone.
KGM + Ca^{++}	KGM+ 0.1mM Ca^{++}

Table 3.1 A list of the various media used.

3.3.4 Western Blot Analysis

Cells were grown in 80cm^2 cell culture flasks (Nunc™, VWR International) in KGM and AFM for a minimum of 5 days and two passages. After washing with PBS, 150µl of lysis buffer (Appendix 1) containing protease inhibitors (Sigma-Aldrich Company Ltd.) was added to each flasks which were then left on ice for one hour. Cells were scraped off using cell scrapers (Nunc™, VWR International) and collected in 1.5ml microtubes (Sarstedt Ltd.). Protein assay (see section 2.3.3.1) was carried out to standardize the amount of protein to be loaded on the gels. SDS polyacrylamide gel electrophoresis was carried out as previously described (section 2.3.3). Mouse anti-E-Cad (Clone HECD-1, Abcam Ltd., Cambridge, UK) was used as a primary antibody at a concentration of 1.8µg/ml, and goat anti-mouse HRP (Biosource, Nivelles, Belgium) was used as secondary antibody.

3.3.5 Indirect Immunofluorescence

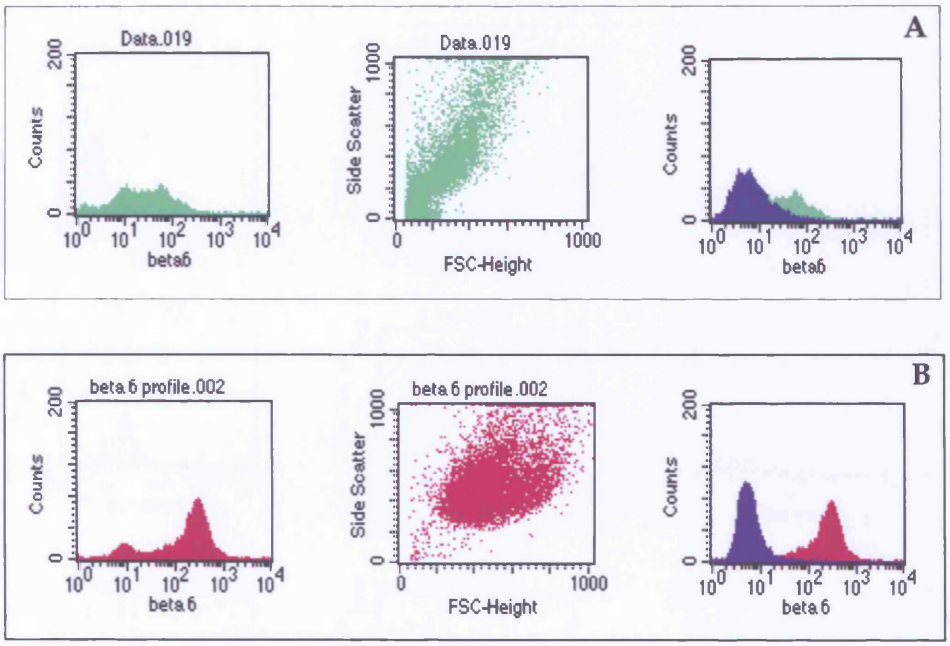
VB6 cells grown in KGM and AFM were seeded in defined keratinocytes SFM at a concentration of 10^4/ml on glass cover slips in a 24-well plate. Cells to be stained for αvβ6 integrin were left overnight at 37°C and those to be stained for E-Cadherin for 48hrs at 37°C. The primary antibodies used were mouse anti-E-Cad (Clone HECD-1, Abcam Ltd.) at a concentration of 1.8µl/ml, and mouse anti-β6 integrin (R6G9, Chemicon International, Chandler's ford, UK) at a concentration of 10µg/ml. The experiment was carried out as previously described (section 2.4.1).

3.4 RESULTS

3.4.1 Characterization of integrin expression by various cell lines

The cells used in this study were analysed by performing a baseline integrin profile. Prior to sorting, VB6 cells showed weak expression of β6 integrin. The FACs analysis showed two peaks with a wide base indicating a wide range of β6 expression of weakly expressing cells and strongly expressing cells. This was resolved after sorting as shown by a clear peak of positive expression (Figure 3.2).

C1 and VB6 were profiled for their α5β1 and αvβ6 integrin (for FACs analysis see Appendix 2). Whenever possible normal human keratinocytes (NHK) were also profiled and when unavailable the normal keratinocyte cell line UP, which has a similar phenotype, was used (Pei *et al.* 1991). Expression of the α5β1 integrin was similar in NHK, C1 and VB6, whereas expression of the αvβ6 integrin was low in C1 and NHK but high in VB6 (Figure 3.3, 3.4, 3.5 and 3.6).

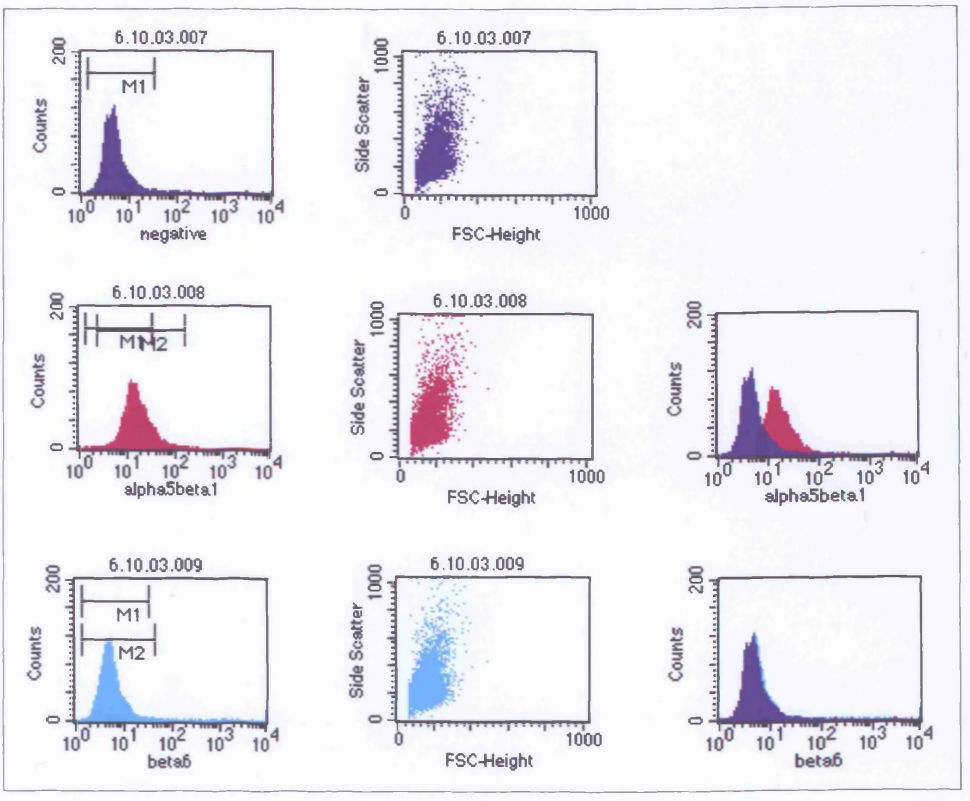


β6 integrin expression in VB6 prior to sorting

β6 integrin expression in VB6 after sorting

Negative control

Figure 3.2 Beta-6 integrin expression in VB6 cells. The concise dot plots denote homogeneity in the cell population. In the first graph the green area denotes the presence of a mixed population of VB6 cells, some expressing β6 and some having lost such expression seen as the part of the green area overlapping with the negative control. This was evident prior to sorting (A). Following sorting the FACs analysis in the lower graphs shows a clear area of cells exhibiting positive β6 expression (pink) (B).



Negative control

α5β1 integrin expression

αvβ6 integrin expression

Figure 3.3 FACS analysis showing α5β1 and αvβ6 integrin expression in C1 cell line. The concise dot plots denote homogeneity in the cell population. In the histograms the area pertaining to the negative control (blue) is measured as M1 while the areas of positive α5β1 (pink) and αvβ6 (light blue) are measured as M2 and correspond to the population of cells expressing each integrin. When each area is overlapped with the negative control, the relative ratio of cells expressing the integrins can be deduced (M2-M1).

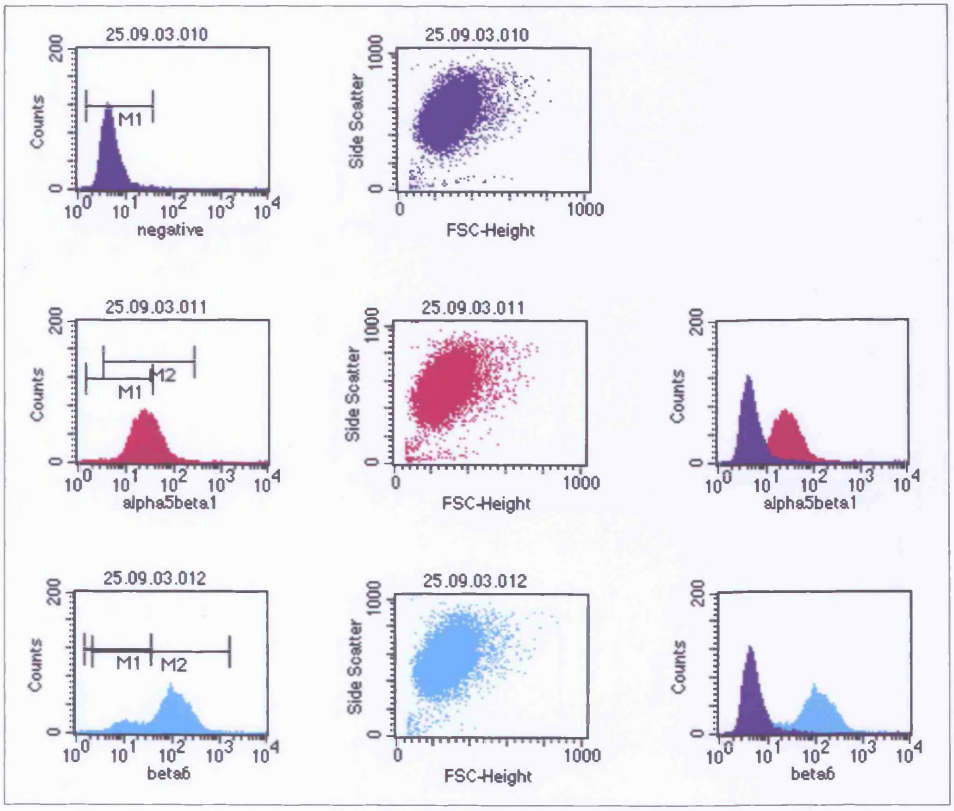


Figure 3.4 FACS analysis showing α5β1 and αvβ6 integrin expression in VB6 cell line.

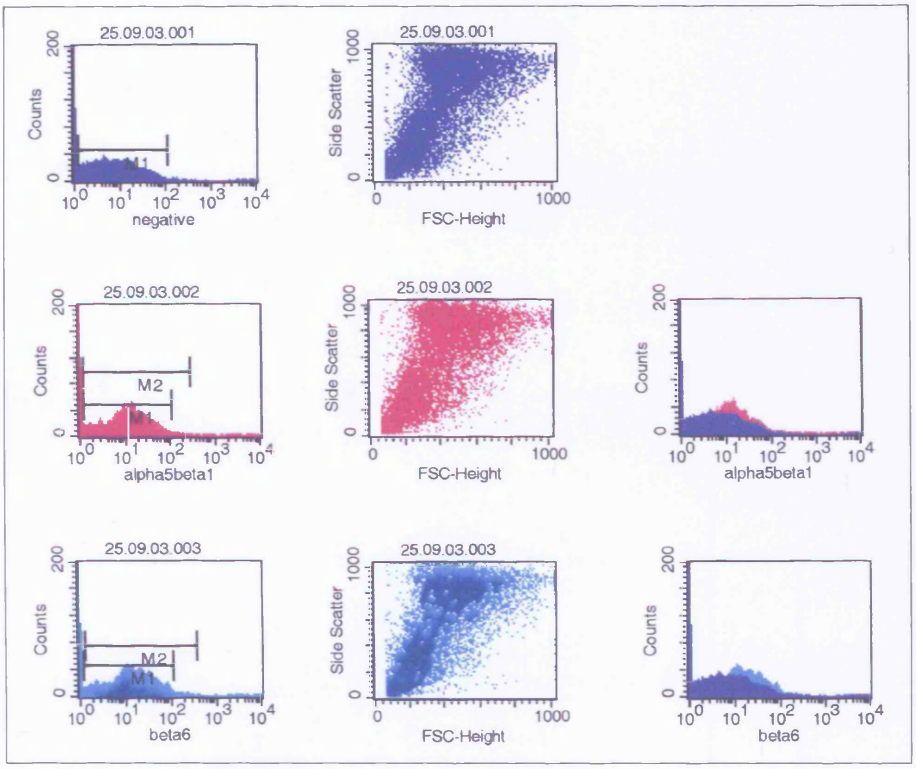


Figure 3.5 FACS analysis showing α5β1 and αvβ6 integrin expression in NHK cell line.

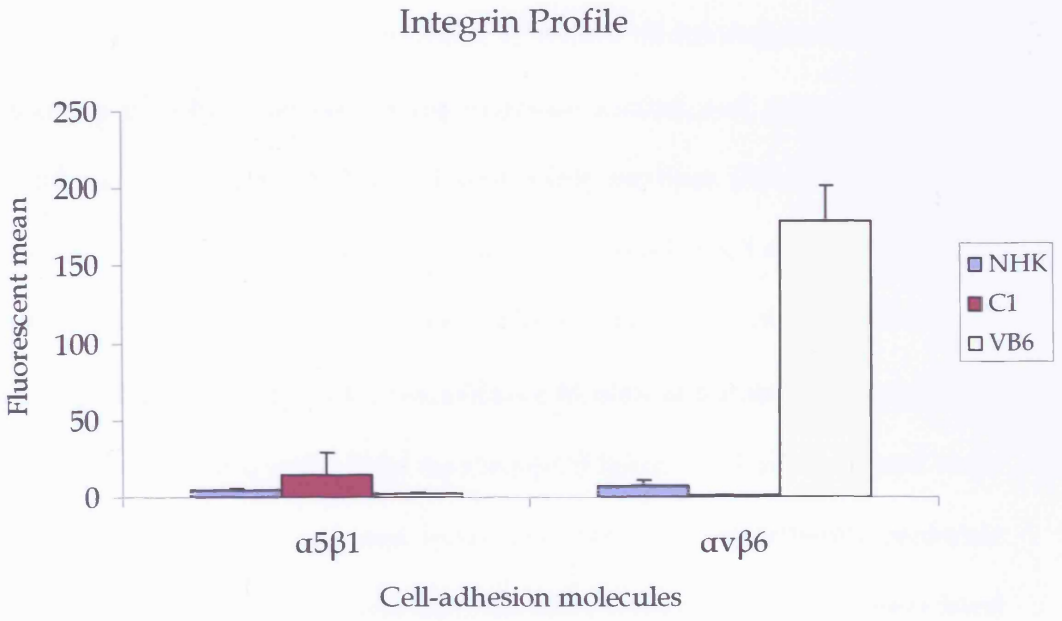


Figure 3.6 Graphic representation of integrin expression in various cell lines.

3.4.2 Morphology of C1 and VB6 changes when grown in growth medium devoid of additives

Keratinocyte growth medium (KGM) contains serum and this in turn contains FN. When testing the possible effect of FN or the fragments on the cell, the FN in the medium posed as a potentially interfering factor. It would be difficult to ascertain the effect of the added FN and fragments under these circumstances. Bearing in mind that cells also have the ability to synthesize endogenous FN, it seemed important to reduce additional sources of FN as much as possible. The cells were therefore seeded and grow the cells in additive-free medium (AFM) and serum-free medium (SFM) to reduce the effect of unwanted exogenous FN to as low as possible. C1 and VB6 grown in SFM and AFM, as opposed to those grown in KGM, were observed to exhibit a changed morphology and a resemblance to normal human keratinocytes in their growth pattern under light microscopy (Figure 3.7). Cells appeared more epithelioid in their growth and spreading pattern. A cell-adhesion molecule profile was run to confirm whether this morphologic change was associated with a change in cell-adhesion molecules expression. Flow Cytometry analysis showed a very subtle difference in E-cadherin profile (Figures 3.8, 3.9 and 3.10). Additives were added one at a time to observe when the cells reverted back to their original form. Normal morphology was restored when EGF was added back to the medium (Figure 3.7 (C)). Western blot for E-cadherin was carried out on lysates of VB6 grown in KGM and AFM (a protein assay was carried out previously to ensure equal protein loading on

the gel, Appendix 2). Results showed clear bands for E-cadherin in the AFM-grown VB6 lysate wells and no bands in the KGM-grown wells (Figure 3.11). This is indicative of some degree of E-cadherin upregulation when grown in additive and serum deprived media.

Indirect immunofluorescence studies were also performed targeting the β6 integrin and E-cadherin on VB6 plated on coverslips grown in AFM and KGM. The β6 integrin staining intensity and distribution showed no difference between VB6 grown in KGM or AFM (Appendix 2). However, E-cadherin staining was more widespread and more intense in VB6 grown in AFM than those grown in KGM (Figure 3.12 and 3.13).

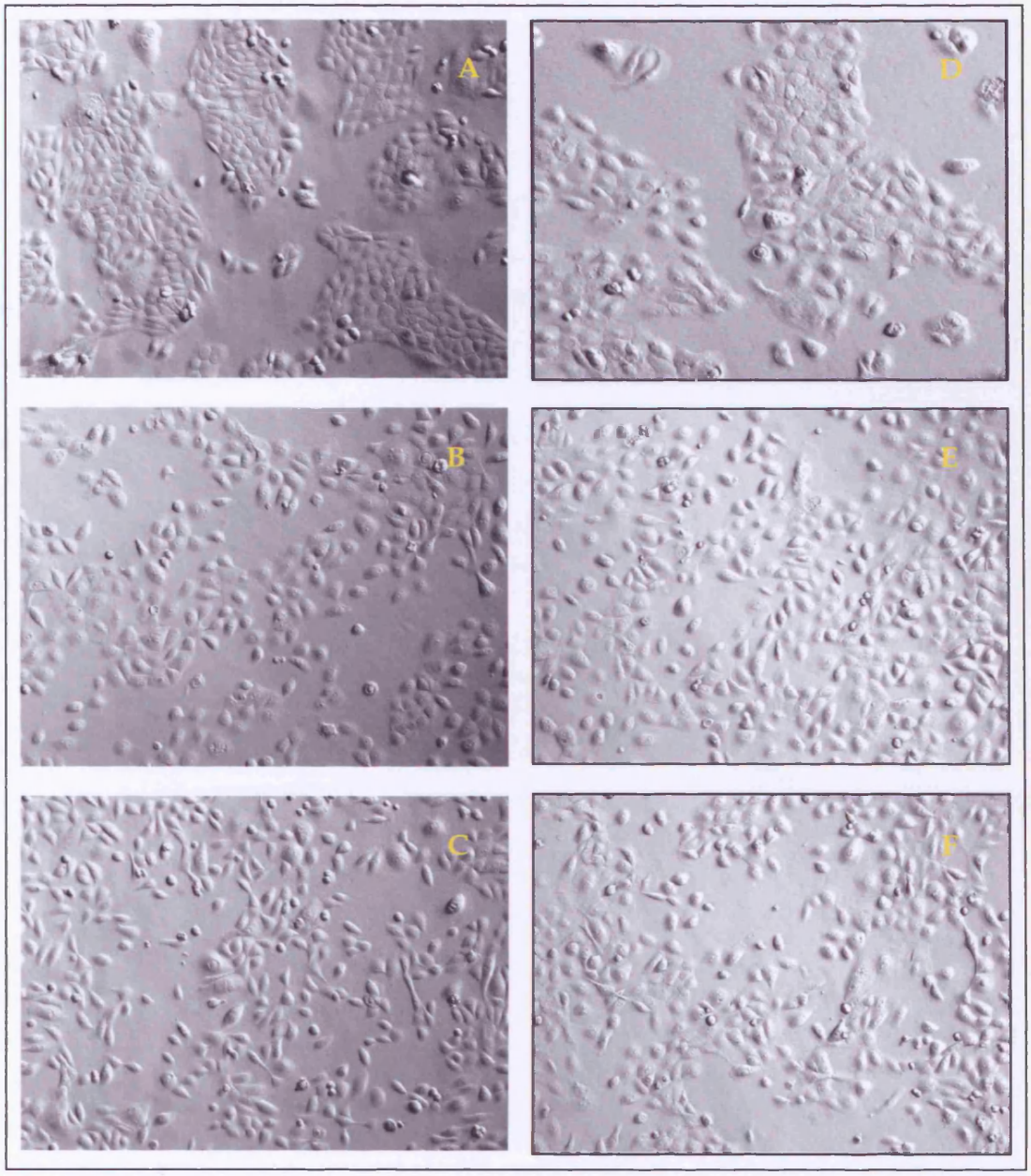


Figure 3.7 Two experiments showing VB6 grown in SFM, KGM and SFM with added EGF (A,B and C); and VB6 grown in AFM devoid of additives; in KGM and AFM with added EGF (D, E and F).

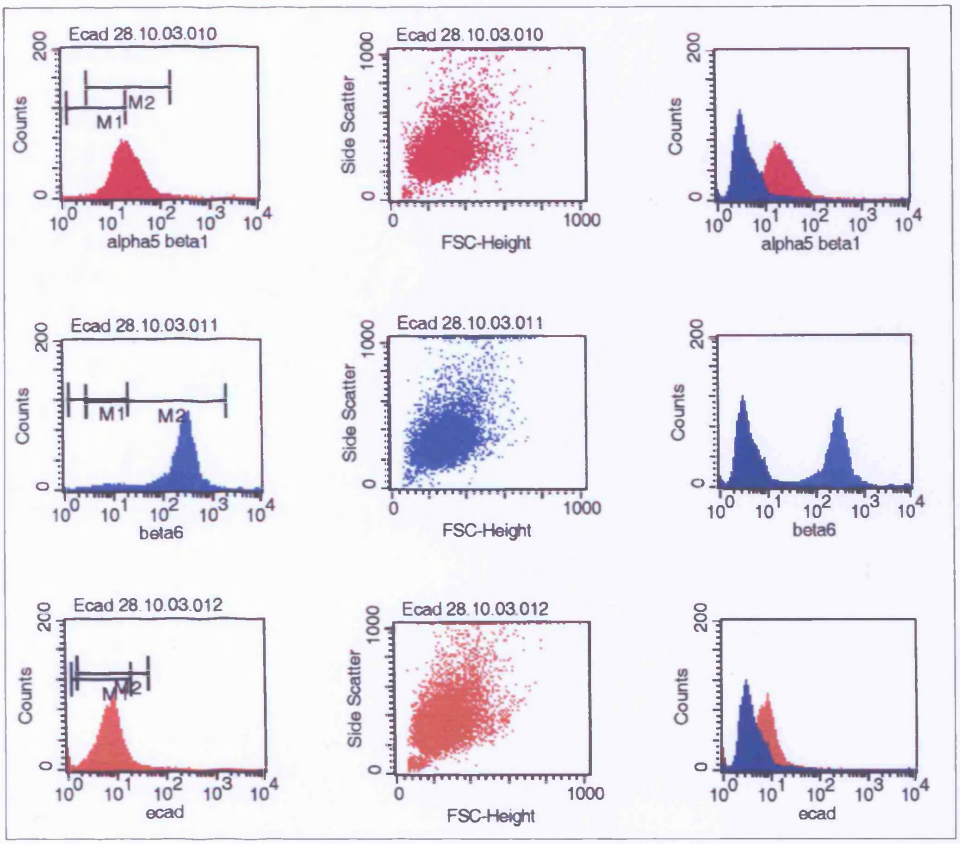


Figure 3.8 FACS analysis showing α5β1 and αvβ6 integrin and E-cadherin expression in VB6 cells grown in KGM.

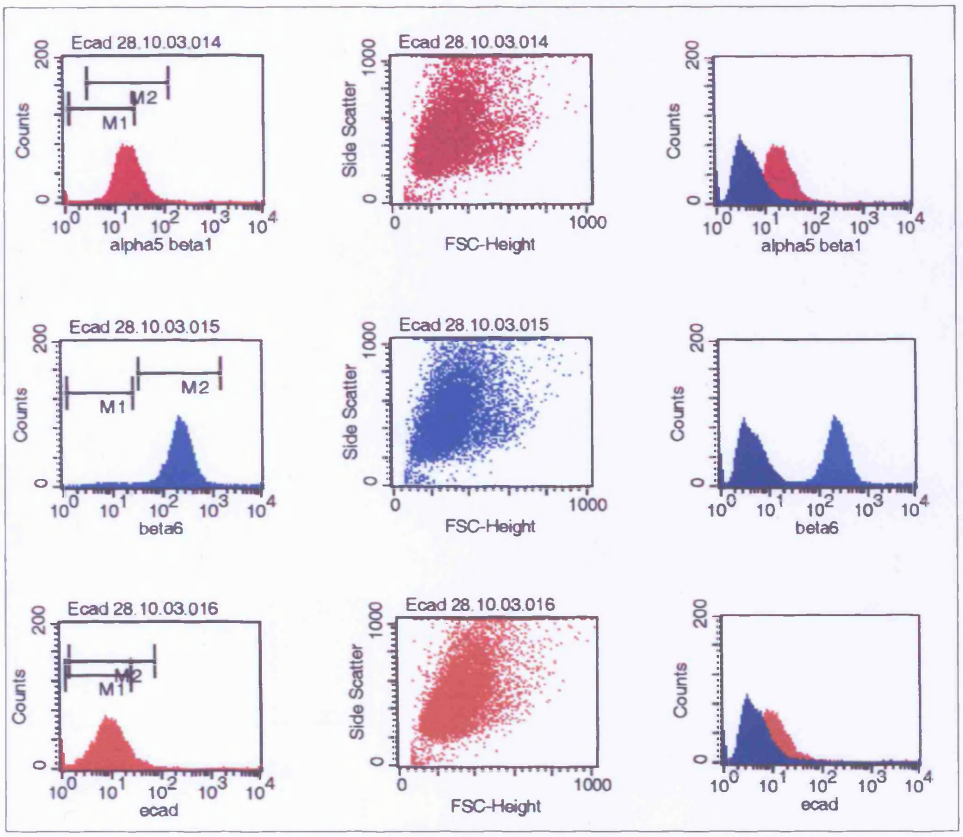


Figure 3.9 FACS analysis showing α5β1 and αvβ6 integrin and E-cadherin expression in VB6 grown in AFM.

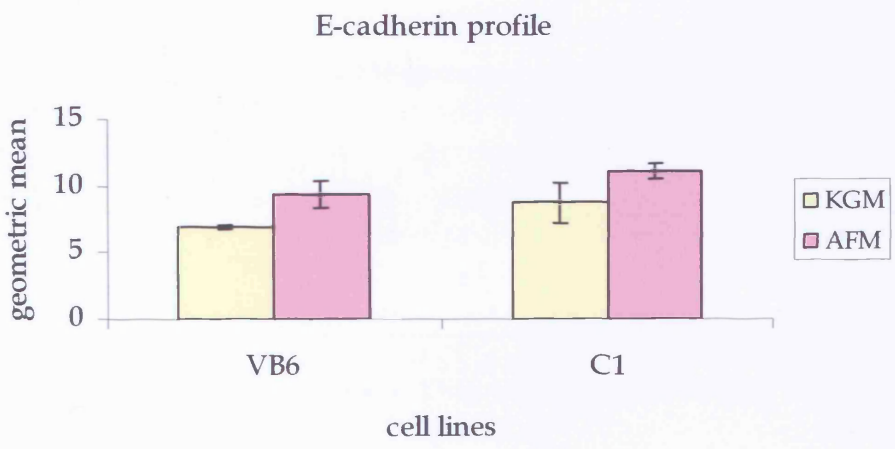


Figure 3.10 Graphic representation of the expression of E-cadherin in VB6 and C1 grown in KGM and AFM from FACS analysis (Figure 3.9 and 3.10).

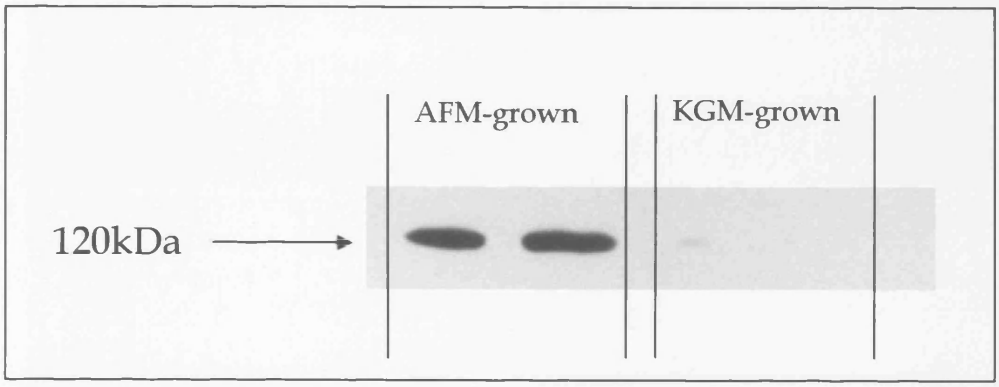


Figure 3.11 Western blot analysis of 15ug total protein showing clear bands for E-cadherin in AFM-grown VB6.

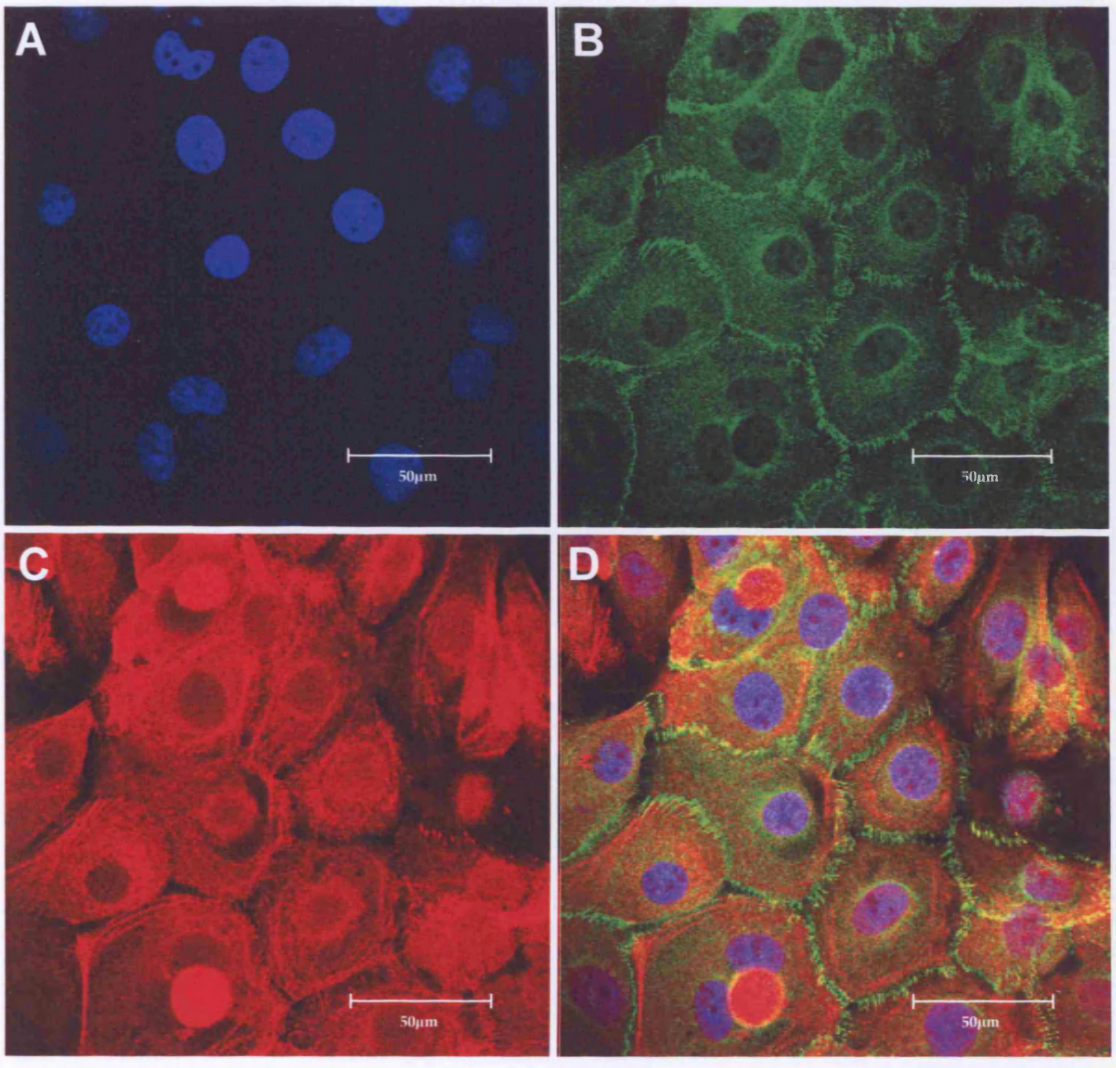


Figure 3.12 Indirect immunofluorescence staining of E-Cadherin (B) and actin (C) and a composite (D) showing obvious cell-cell adhesion in VB6 grown in AFM.

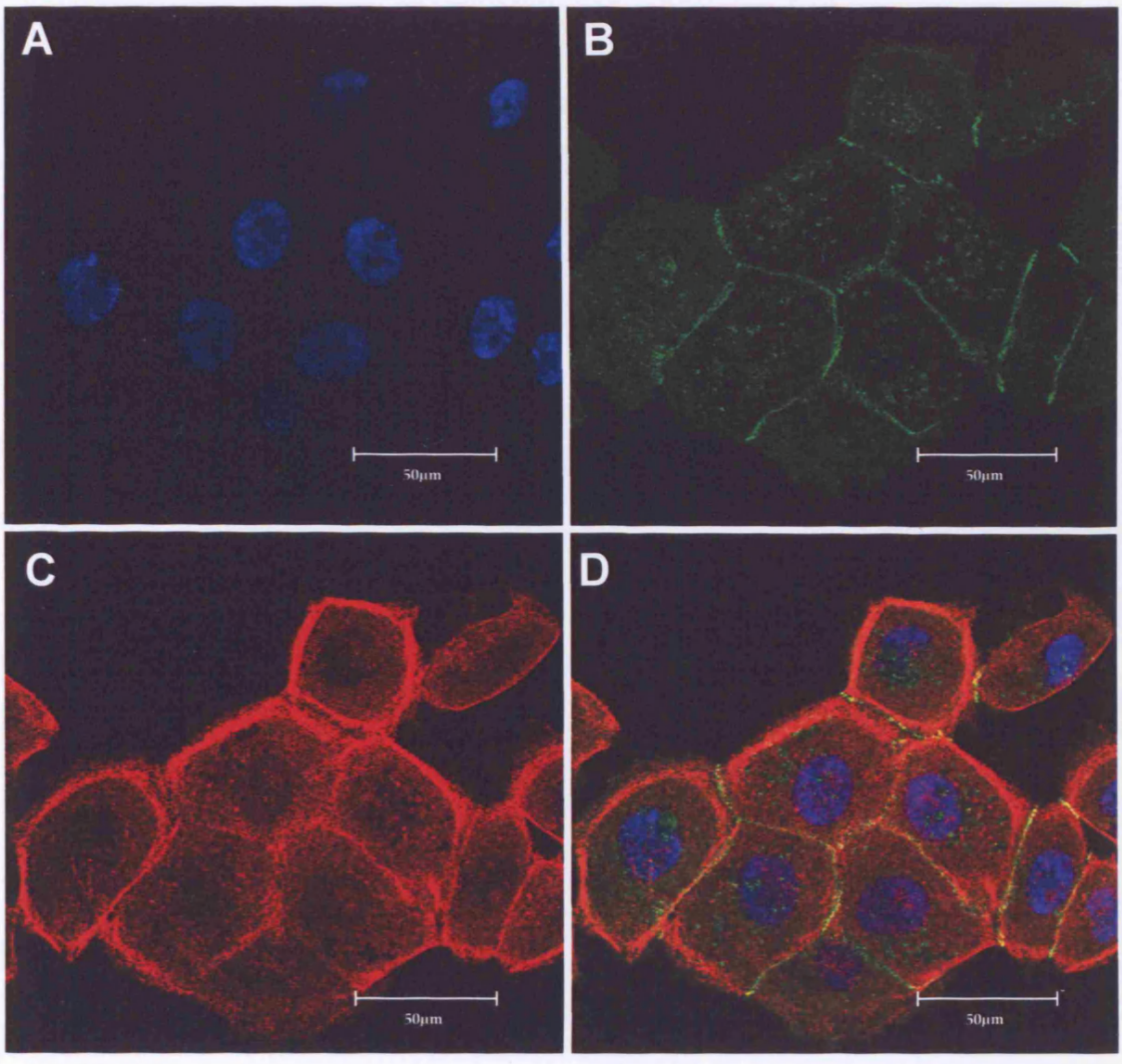


Figure 3.13 Indirect immunofluorescence staining of E-Cadherin (B) and actin (C) and a composite (D) showing less cell-cell adhesion in VB6 grown in KGM.

3.5 DISCUSSION

During wound healing and tumourigenesis cells exhibit an alteration in their cell adhesion molecule repertoire. Integrins and cadherins are among such molecules whose expression indicates the nature of the cells. Both cell lines used in this study express the α5β1 integrin in similar amounts (Figure 3.5 and 3.6) (Thomas *et al.* 2001a). FACS analysis of our cell lines revealed the expression the α5β1 integrin to be similar in both VB6 and C1 but low in NHK. Expression of αvβ6 was low in both NHK and C1 and high in VB6. This concurred with previous studies (Thomas *et al.* 2001b; Ramos *et al.* 2002).

3.5.1 Medium manipulation and cell morphology

Cells respond to environmental prompts and the smallest fluctuations in factors such as media content, homogeneity of the cell populations or seeding densities can lead to altered effects. Throughout this work a diverse number of parameters were tested including variations in cell seeding densities, growth media and mode of application of fragment, either as a coating or in suspension. In this section the homogeneity of the cell lines was assessed to ensure their expression of specific integrins. Furthermore, the effects of various media on the cells' essential characteristics was also assessed.

The ECM surrounding all living cells signals to them via its structural molecules or by harbouring growth factors or growth factor-binding proteins.

Any alterations in the ECM structure or contents can therefore have profound effects on cells' differentiative and homeostatic mechanisms (Streuli 1999). Previous work by Altanov *et al.* (2001) showed that cells grown in serum-free medium (SFM) express reduced ability to organize a FN matrix. This group found that growing dermal keratinocytes in SFM changed their morphology to a smaller and less polarized one. Changing the medium reversed this and induced cells to grow in colonies. Medium manipulation therefore plays an important role in keratinocyte morphology and FN matrix assembly. SFM grown cells seem to exhibit a more stationary type and seem to adhere more strongly to the FN matrix. However, when switched to serum-containing medium such stationary cells seemed more likely to move along and degrade the FN. This group suggests that the way keratinocytes are cultured may affect not only their morphology but also their migratory potential and behaviour (Altanov *et al.* 2001).

While assessing the effect of medium-manipulation on our cells, a change in cell morphology was observed when the cells were starved of additives normally present in keratinocyte growth medium. Both VB6 and C1 showed a change in morphology from rounded, evenly-growing, mostly single cells to epithelioid, island-growing cells, resembling normal human keratinocytes. Since both integrins and cadherins play pivotal roles in regulating diverse processes such as differentiation, morphology and cell migration, it is likely that these two cell adhesion molecules act co-ordinately to regulate such

processes. This has been shown in keratinocytes where cadherins down-regulate integrin expression during keratinocytes differentiation but not *vice versa* (Hodivala & Watt 1994). It seems likely that the missing additives somehow switched the cells to exhibit more E-cadherin, or that the E-cadherin already present was more active. A cell-adhesion molecule profile was run investigating α5β1, αvβ6 integrins and E-Cadherin expression in cells grown in KGM and in AFM. Both integrins' expression remained unchanged in VB6 grown in KGM and AFM. However, our results suggest a slight upregulation of E-cadherin in cells grown in AFM. To confirm this, Western blot analysis of lysates of VB6 grown in KGM and AFM was carried out and showed E-cadherin expression in cells grown in AFM but not in those grown in KGM. Indirect immunofluorescence showed more intense E-cadherin staining in cells grown in AFM. Together the results observed suggest the presence of a "cadherin switch" brought about by manipulating the medium.

3.5.2 Integrin-cadherin switch

The change in morphology seen when growing cell in AFM could also be explained by an integrin-cadherin switch where the upregulation of one causes the down regulation of the other and vice versa. Weaver *et al.* (1997) showed that changes in tissue structure were critical for the expression of the malignant phenotype and ECM receptors are important modulators of these effects. An integrin switch where a cell may change its integrin expression pattern may enhance the survival of cancer cells in changing environments

and encourage metastasis. Hodivala *et al.* (1994) suggested a likely cross-talk between cell adhesion molecules whereby cultured keratinocytes lacking cadherin adhesion showed decreased overall expression of integrins and *de novo* expression of some (such as αvβ6) while undergoing differentiation. Absence of E-cadherin was also found to correlate with extended filipodia processes joining other cells (Hodivala & Watt 1994; Chunthapong *et al.* 2004). Huttenlocher *et al.* (1998) elegantly proposed a synergistic dialogue between these two cell adhesion molecules stating that while cadherins are necessary for the termination of motility in a cell, they are not sufficient. Their observation showed that alterations in integrin expression can result in changes in cadherin expression and changes in its function. Seeing that cadherins themselves may be involved in the down-regulation of integrin expression, a loop is formed linking the two molecules together in a "tug-of-war" (Hodivala & Watt 1994).

3.5.3 EGF and cell morphology

Systematic reintroduction of the various additives was carried out and images were taken under light microscopy (Appendix 2). When EGF was reintroduced in AFM, the cells reverted to their original tumour-like growth and morphology. This was irrespective of the presence or absence of serum in the medium (similar experiments were carried out with SFM, Appendix 2). Many reports have associated the EGF receptor (EGFR) to E-cadherin expression. Over-expression of the EGFR family members strongly correlates

with poor prognosis in carcinomas (Hazan & Norton 1998; Al Moustafa *et al.* 1999). This inverse relationship is in accord with the strong correlation between the loss of E-cadherin and enhanced cell invasiveness in vitro and tumour progression in vivo (Hazan & Norton 1998; Pece & Gutkind 2000). Al-Moustafa *et al.* (1999) observed an inverse relationship between EGFR activation and E-cadherin expression. Activation of EGFR was found to affect the adhesive function of E-cadherin via the β-catenin pathway. When EFGR inactivating antibodies were added to cultures, expression of E-cadherin was upregulated and the cells changed to a more epithelial phenotype as shown by indirect immunofluorescence and flow cytometry (Al Moustafa *et al.* 1999). This group further demonstrated that expression and modulation of EGFR is essential for phenotype conversion and proliferation of human epithelial-like lung cancer cells. Furthermore, in the same lung cancer cell line EGF was able to produce cell scattering possibly affecting motility; EGF-antibodies induced upregulation and re-localization of E-cadherin/catenin complex (Al Moustafa *et al.* 2002). This is likely considering that EGFR is co-localized to adhesive structures with E-cadherin even in the very early stages of cell-cell adhesion complex formation. Both E-cadherin and EGFR cooperate directly to activate signalling pathways (Pece & Gutkind 2000).

In our work the cells reverted to their original phenotypic appearance by simply adding EGF back into the medium without carrying out any other manipulation; it seems that the mechanism involved could be very simple and

basic. The reversion was observed to be irrespective of the cells' expression of the integrins associated with high motility and aggressiveness, portent of the secondary role played by such integrins in this situation. It has been reported that changes in cadherin expression alter integrin expression and therefore affect differentiation (Huttenlocher *et al.* 1998). Hodivala and Watt (1994) provided evidence that E-cadherin is upregulated during keratinocyte differentiation. Deugnier *et al.* (2002) showed that mammary basal epithelial cells, displaying phenotype plasticity, grown in EGF deficient medium had good cell-ECM contacts and lacked good cell-cell contacts. This was reversed by growing the cells in EGF containing medium for 8-10 days. It seems that EGF acts as a negative influence of basal keratinocyte phenotype and this appears to be a reversible effect. Treatment of mammary basal epithelial cells with EGF induced the production of MMP-2 and increased scattering and mobility on laminin-5 (Deugnier *et al.* 2002). This group grew cells with and without EGF and the former, while retaining the same integrin repertoire, showed an upregulated secretion of MMPs, particularly MMP-2. This was also reflected in the cells ability to migrate over laminin-5. Integrins and cadherins are both connected to the cell's cytoskeleton and the theory is that they may compete for some accessory cytoskeletal molecule that is required for stable expression during keratinocyte differentiation (Hodivala & Watt 1994). Yet others have shown that squamous carcinoma cells, despite retaining E-cadherin expression differed from the non-malignant counterparts in their expression of integrins, intermediate filaments and protease secretion

(Ghosh *et al.* 2002). This inverse relationship was not seen in normal keratinocytes (Gasparoni *et al.* 2004). Although links between E-cadherin and integrins have been previously shown, alterations in the level of E-cadherin induced by media components in our data did not result in changes in α5 and β6 integrin expression.

3.5.4 Conclusion

Loss of specific components of the ECM, changes in ECM receptors, growth of cells on different types of ECM and in different types of media have all been found to affect the differentiation of specific cell types (Bissell & Nelson 1999). Tumour cells thrive on the signals they receive from their surroundings and EGF is a major component of tumour microenvironment. E-cadherin and integrins are important determinants of cell behaviour and their expression was shown to be influenced by medium constituents. A loop seems to exist connecting integrins, E-cadherin and EGF, and is liable to include more molecules and signalling pathways as more associations are established.

Signalling events linked to the maintenance of normal tissue architecture are sufficient to abrogate malignancy and to repress the tumour phenotype (Weaver *et al.* 1997). The malignant cell's inherent ability to secrete and attract the molecules it needs to sustain it, enables it to become resistant to the body's natural defences and becomes immortalized. Adding or omitting additives in

the medium sustaining cancer cells in vitro may therefore alter the cell's phenotype and characteristics.

CHAPTER 4

FIBRONECTIN FRAGMENTS AND CELL MOTILITY

4.1 INTRODUCTION

Cell motility is an essential step in cancer progression. In order for cancer cells to spread and metastasize they must first detach from the primary lesion, breach the basement membrane and remodel the surrounding extracellular matrix in order to reach distant loci. Cancer cells are able to alter their expression of cell adhesion molecules and production of proteases in order to enable them to accomplish such migration. This complex mechanism involves the interaction of tumour cells with a myriad of extracellular matrix molecules and their fragments and this is mainly mediated through integrins. Upon binding of integrins to various ligands a cascade of signalling is initiated that not only alters the gene expression but also initiates association with the cell cytoskeleton to promote cell motility and cell-attachment/detachment.

4.1.1 Integrins and cell motility

The role of integrins as bidirectional signal transducers makes them central to the search for therapies that target and alter their signals. Many studies have shown the altered expression of various integrins during tumour growth and progression (Clezardin 1998; Mizejewski 1999) in particular the integrins involved in cell motility; such integrins are either over-expressed or no longer expressed. Although most tumours retain normal expression of at least some

of their integrins, some integrins may also be selectively activated/inactivated to avoid cell adhesion and extracellular matrix binding at inconvenient times and locations (Mizejewski 1999). Integrins are repeatedly implicated in malignant transformation, tumour progression and metastasis (Mizejewski 1999). Of particular interest to our study are changes in the expression of FN-binding integrins, α5β1 and αvβ6. Generally the α5β1 integrin correlates with low level of transformation (Clezardin 1998; Mizejewski 1999) and its re-expression has a negative effect on anchorage-independent growth (Clezardin 1998). The αvβ6 integrin is expressed *de novo* in tumours and during wound healing and is also thought to play a role in the transition to a malignant phenotype (Niu *et al.* 2001). The upregulation of the αvβ6 integrin by tumour cells makes it a potential marker of malignancy and aggressiveness.

4.1.2 Fibronectin Fragments and cell motility

The extracellular matrix is far from an inert unchanging scaffold, but rather is a rich dynamic information-rich source of inputs for cells (Hynes 1992; Hynes 1999). It seems probable that fragments of extracellular matrix molecules provide motility cues in the tumour microenvironment. Digestion of these molecules by various proteases might expose otherwise hidden cryptic sites that possess activities quite different from the parent molecule (Yamada 2000). Fibronectin is an important extracellular matrix component that has been associated with normal cell phenotypes. Fibronectin-rich matrices provide substrates for cell adhesion and migration during development, wound

healing and tumourigenesis (Hynes 1992; Hynes 1999). During tumour transformation, one of the first observations is the failure of tumour cells to deposit such a FN matrix (Colombi *et al.* 2003). It has been proposed that the fragmentation of FN is brought about by the up-regulation of protease secretion and down-regulation of FN-binding proteins in cancer cells (Colombi *et al.* 2003). Various FN fragments have been studied in relation to migration and protease secretion.

4.2 OBJECTIVES

To assess the effect of FN fragments on migration, adhesion and the morphology of VB6 and C1 cells when compared to the intact molecule.

4.3 MATERIALS AND METHODS

Based on results from our previous chapter, defined SFM (Invitrogen Corporation, Gibco™) was our growth medium of choice during our experiments, unless otherwise stated.

4.3.1 Migration Assays

Haptotactic cell migration was measured using polycarbonate Transwell® inserts as described in section 2.2.1 (Corning Costar). The underside was coated with pFN, 120 kDa FN fragment (Upstate®, Biotechnology, Botolph Clayden, UK), 30 kDa amino-terminal FN fragment (Sigma-Aldrich Company Ltd.) and PHSRN peptide (Upstate®, Biotechnology) diluted in PBS to give a final concentration of 1μM/ml, and the cells were allowed to migrate over a period of 4 hours (Figure 4.1).

Figure 4.1 High power microscope image of a field from a Transwell® membrane mounted on a slide. Cell (arrow) were counted per field.

In the blocking experiments, mouse anti-human α5β1 monoclonal antibody P1D6 and mouse anti-human αvβ6 monoclonal antibody 10D5 (Chemicon International, Chandler's ford, UK) were used at a concentration of 10μg/ml.

4.3.2 Adhesion Assays

Using a 96 well plate triplicate wells were coated with 100μl of pFN, 120 kDa fragment and 30 kDa fragment, diluted in PBS to give a final concentration of 1μM/ml, for one hour at 37°C, as previously described (section 2.2.3). Doubling standard dilution of cells was set up in SFM. 20μl of MTS (Cell Titer 96® Aqueous One Solution Cell proliferation Assay, Promega UK) was added to both plates and following one hour incubation at 37°C in the dark, the absorbance was measured at 490nm by using Plate Reader 340 ATTC (SLT Labinstruments, Austria).

4.3.3 Wound Assays

VB6 and C1 cells were grown to just below full confluence, trypsinized and seeded at 10^5 in 24 well plates. In one set of triplicate experiments the wells were *coated* with 250μl of pFN or 120 kDa FN fragment in PBS to give a final concentration of 1μM/ml. In another set of triplicate experiments, rather than coating the wells, the cells were seeded in uncoated wells but were *incubated* with defined SFM (Invitrogen Corporation, Gibco™) containing the various matrix proteins at dilutions that gave the same final concentration as

mentioned previously. The experiment was carried out as previously described in section 2.2.2 (Figure 4.2).

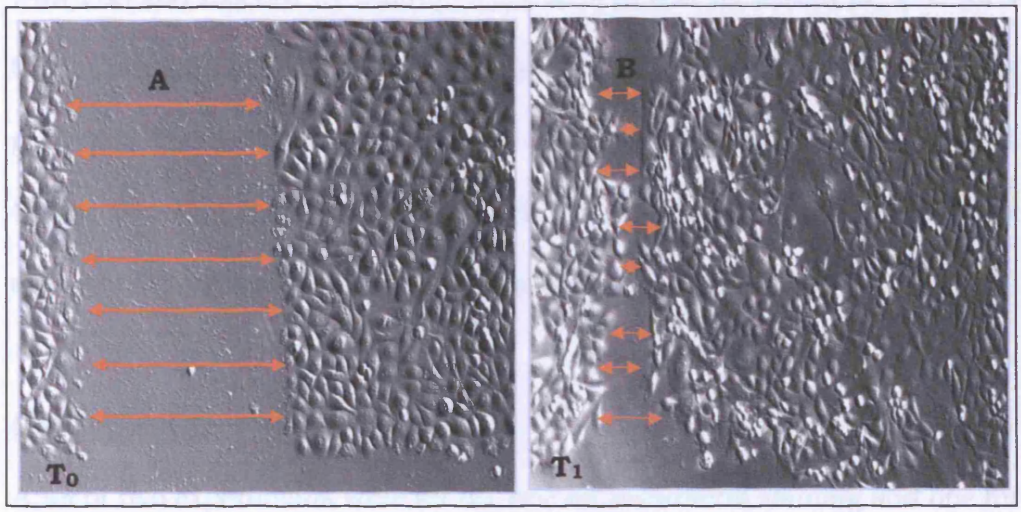


Figure 4.2 High power images of a wound assay at T_0 and T_1. At T_0 various random measurements are taken at various points. At T_1 the same area is visualized and random measurements are taken again. Means are then calculated for both A and B and the mean for T_1 is subtracted from T_0. The result is an indication of the average distance migrated by the cells over time to close the wound.

4.3.4 Proliferation Assays

To establish the optimal cell density to seed in each proliferation assay experiment, a pilot experiment was carried out using C1 and VB6 at two densities, ($15x10^4$ and $30 x10^4$) and using two time points, T_0 and T_1 (after 24 hours). We established $30 x10^4$ to be the optimum to use in all the following experiments (Appendix 2). Triplicate wells in a 96 well plate were coated with

pFN and 120 kDa fragment diluted in PBS at a final concentration of 1μM/ml and the control being PBS. One plate was set with standards of cell densities starting from $6x10^4$. Four plates were set up; T_0 being 6 hours, T_1 24 hours, T_2 48 hours and T_3 76 hours. At each specified time point the wells were washed with PBS and blocked by adding 100μl of SFM and incubated at 37°C for 1 hour. Twenty μl of MTS (Promega UK) was then added to each well and the plate was again incubated at 37°C for 1 hour. Absorbance was measured at 490nm by using Reader 340 ATTC (SLT Labinstruments). The number of adherent cells was calculated by comparison to the standard curve.

4.3.5 Indirect Immunofluorescence

A set of two experiments were set up, one for E-cadherin staining and one for αvβ6 integrin staining, both concomitant with actin and nuclei staining. VB6 cells were seeded in defined keratinocyte SFM (Invitrogen Corporation, Gibco™) at a concentration of 10^4 on glass cover slips in a 24-well plate, cells to be stained for αvβ6 integrin were left overnight at 37°C and those to be stained for E-cadherin for 48hrs at 37°C.

Cells were rinsed and washed using 0.1/0.1 solution of 0.1% azide, 0.1% BSA in PBS and primary antibodies added, R6G9 monoclonal mouse anti-β6 at a concentration of 10μg/ml in 0.1/0.1 solution, and anti-E-Cad (Clone HECD-1, Abcam Ltd.) at a concentration of 1.8μg/ml and incubated for 1 hour at room temperature. Negative controls were left in PBS devoid of primary antibody.

Following rinsing and washing off the primary antibodies, the cells were incubated with the secondary antibody for 30 minutes at room temperature and in a dark environment. Actin was stained using phalloidin-TRITC (Sigma-Aldrich Company Ltd.), and nuclei were stained with DAPI (Sigma-Aldrich Company Ltd.). After mounting the cover slips face down onto glass slides, images were captured using a Confocal Inverted Laser Scanning microscope (Zeiss Axiovert 200M, Welwyn Garden City, UK).

4.4 RESULTS

4.4.1 VB6 migration towards fragments: Up-regulation towards the 120kDa FN fragment

Beta 6 expressing cells were found to migrate at approximately twice the rate towards the 120 kDa fragment as compared to the intact pFN, and the 30 kDa FN fragment (Figure 4.3 and Figure 4.4). Migration of these cells towards pFN was also three-fold higher than for the C1 towards the same molecule. This confirms the importance of the αvβ6 integrin in the mediation of such migration despite the presence and known function of the α5β1 integrin in both cell lines. Furthermore, blocking experiments with anti-α5β1 antibody P1D6 and anti-αvβ6 antibody 10D5 showed a significant reduction in migration when the αvβ6 integrin is blocked (Figure 4.5). Blocking both integrins by using both antibodies showed an additive effect and lowered migration further. This clearly outlines the importance of both integrins in cell migration towards FN and in particular the αvβ6 integrin towards the 120kDa FN fragment.

Wound assays showed no significant differences in migration whether the fragments and the parent molecule were coated on the wells or incubated within the culture medium (Figure 4.6 and 4.7), and no significant difference between the VB6 and C1 cell lines.

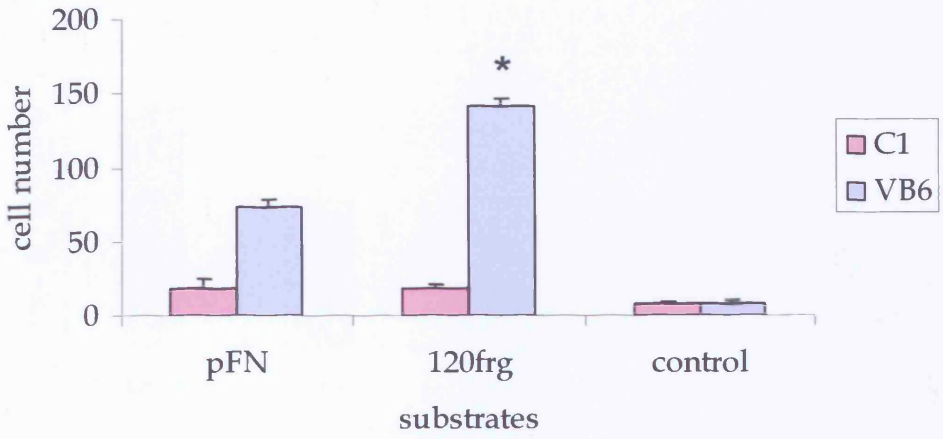


Figure 4.3 Migration of C1 and VB6 towards pFN, 120 kDa FN fragment and control (PBS).VB6 cells showed a significant increase in migration towards the 120kDa FN fragment compared to pFN (p value=0.001), and overall increased migration towards both the fragment and intact molecule compared to C1.

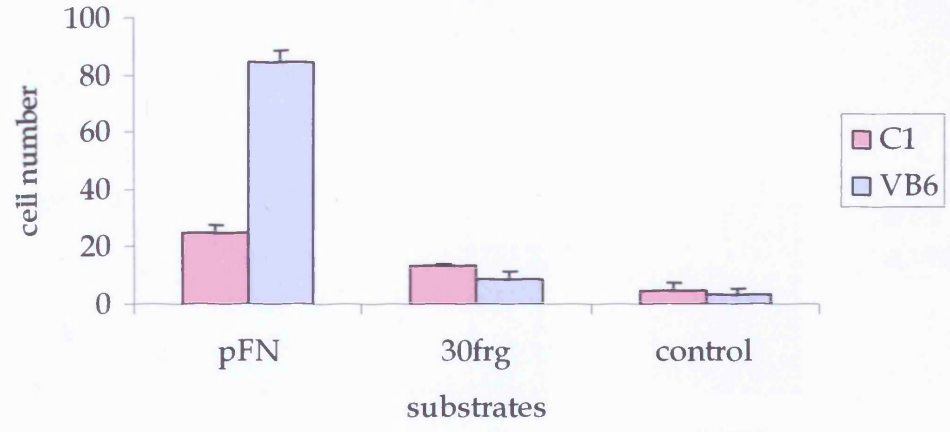


Figure 4.4 Migration of C1 and VB6 towards pFN, 30 kDa FN fragment and control (PBS). VB6 cells showed increased migration towards the intact pFN molecule but failed to show any difference from the C1 in migration towards the 30 kDa FN fragment.

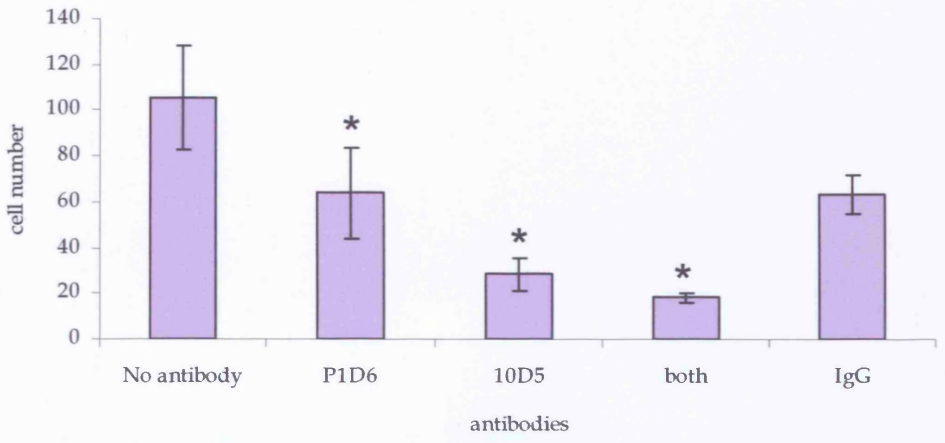


Figure 4.5 Migration of VB6 cell line on 120kDa FN fragment using no antibody, anti-α5β1 antibody (P1D6), anti-αvβ6 antibody (10D5), both antibodies and IgG control . A significant reduction in migration was observed using P1D6, 10D5 and both antibodies together (p value=0.001).

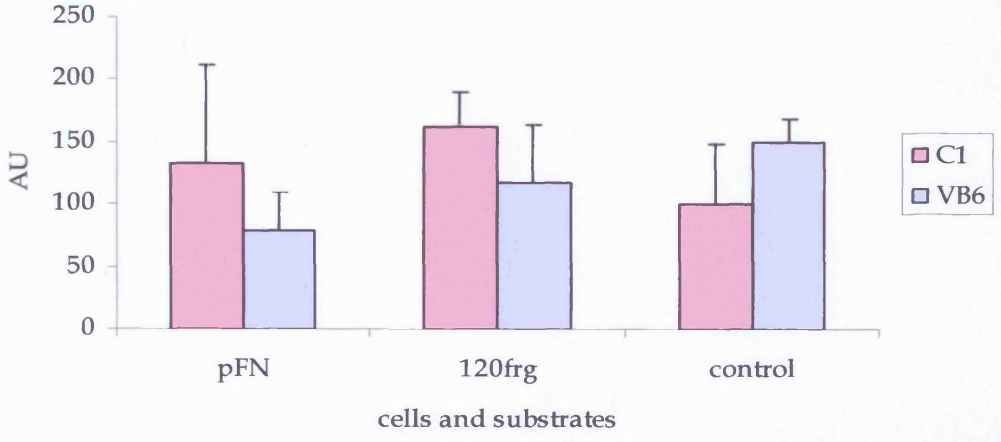


Figure 4.6 Wound assay on plate coated with 120 kDa FN fragment and pFN.

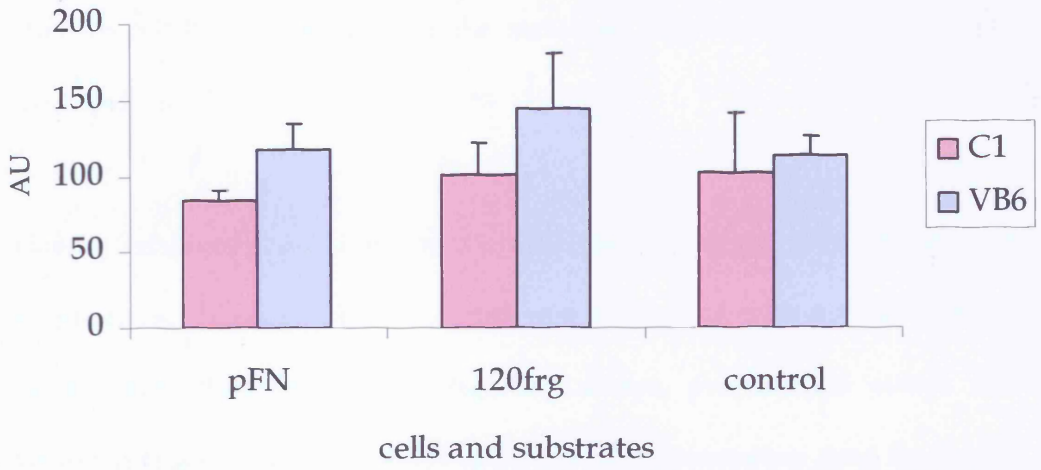


Figure 4.7 Assay showing wound closure of C1 and VB6 incubated with 120kDa FN fragment and pFN.

4.4.2 Increased migration towards the 120kDa FN fragment is independent of attachment and proliferation

In our study we have found no apparent difference in proliferation rate among both C1 and VB6 on pFN and the various fragments (Appendix 2). This indicates that the increased migration is not due to increased proliferation resulting in increased cell number. The similarity in adhesion of the two cell lines indicates that the increased migration is independent of increased initial adhesion (Figure 4.8).

Having established that there was a difference in migration on the various substrates a comparison of the results was carried out with normal primary human keratinocytes (NHK). Migration assays, proliferation assays and adhesion assays were carried out using the same parameters used for the C1 and VB6. The up-regulated migration observed in the VB6 on the 120kDa was absent in the normal cell lines, pFN was in fact more pro-migratory than the fragment (Figure 4.9). Proliferation assays showed no apparent difference among the various substrates (Figure 4.10).

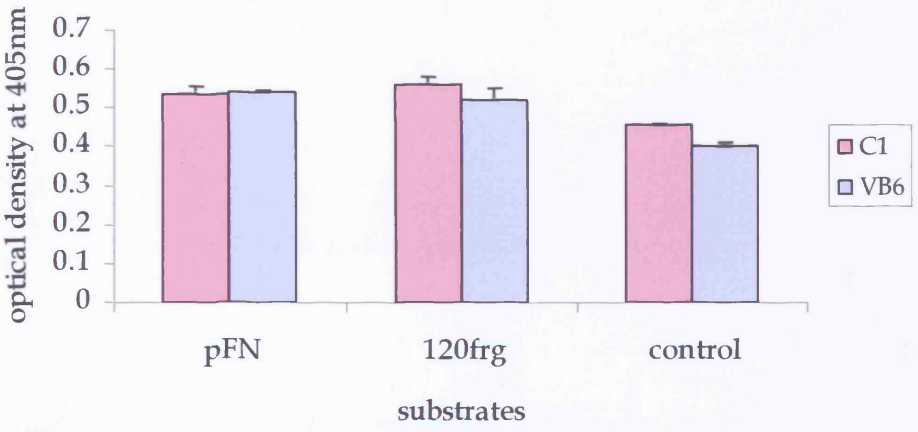


Figure 4.8 Adhesion assay showing adhesion of VB6 and C1 on pFN, 120kDa FN fragment and control.

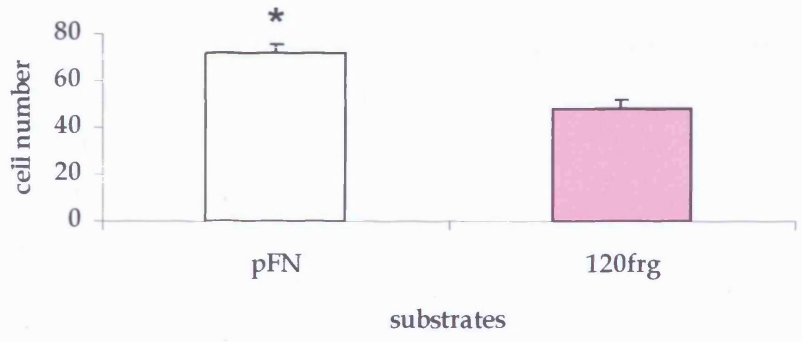


Figure 4.9 Migration of NHK on pFN and 120kDa FN fragment; control was omitted on account of the absence of migration without a substrate. NHK cells showed a significant increase in migration towards the pFN rather than the 120kDa FN fragment (p value= 0.001)

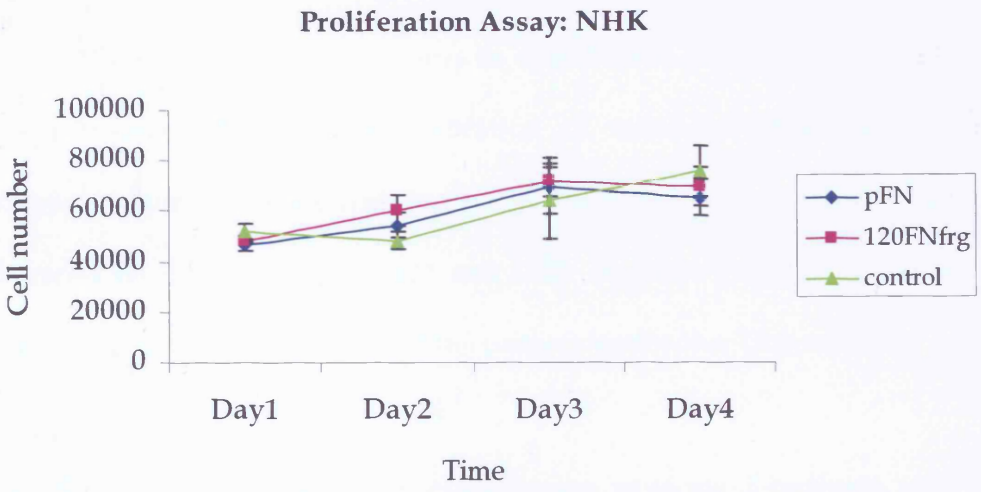


Figure 4.10 Proliferation assay of NHK on pFN and 120kDa FN fragment.

4.4.3 Morphology studies by indirect immunofluorescence of cells plated on various substrates

In order to investigate the effect of the 120kDa FN fragment on cell morphology and compare it to the effect of the intact molecule, indirect staining by immunofluorescence was carried out. Both actin and the αvβ6 integrin of VB6 plated on the two substrates were stained and observed. There is little evidence of any changes in distribution pattern of the αvβ6 integrin, however, there is an indication of increased filopodia and lamellipodia in the cells seeded on the 120kDa FN fragment as opposed to the ones seeded on the pFN (Figure 4.11 and 4.12). Negative controls for these experiments involved the omission of the primary antibodies (Appendix 2).

Immunofluorescent analysis of the cell-adhesion molecule E-cadherin was also carried out to complete the morphologic characterization of the cells on account of its importance in cell morphology and close association to the actin cytoskeleton. No apparent difference in stain intensity was observed (Figure 4.13 and 4.14).

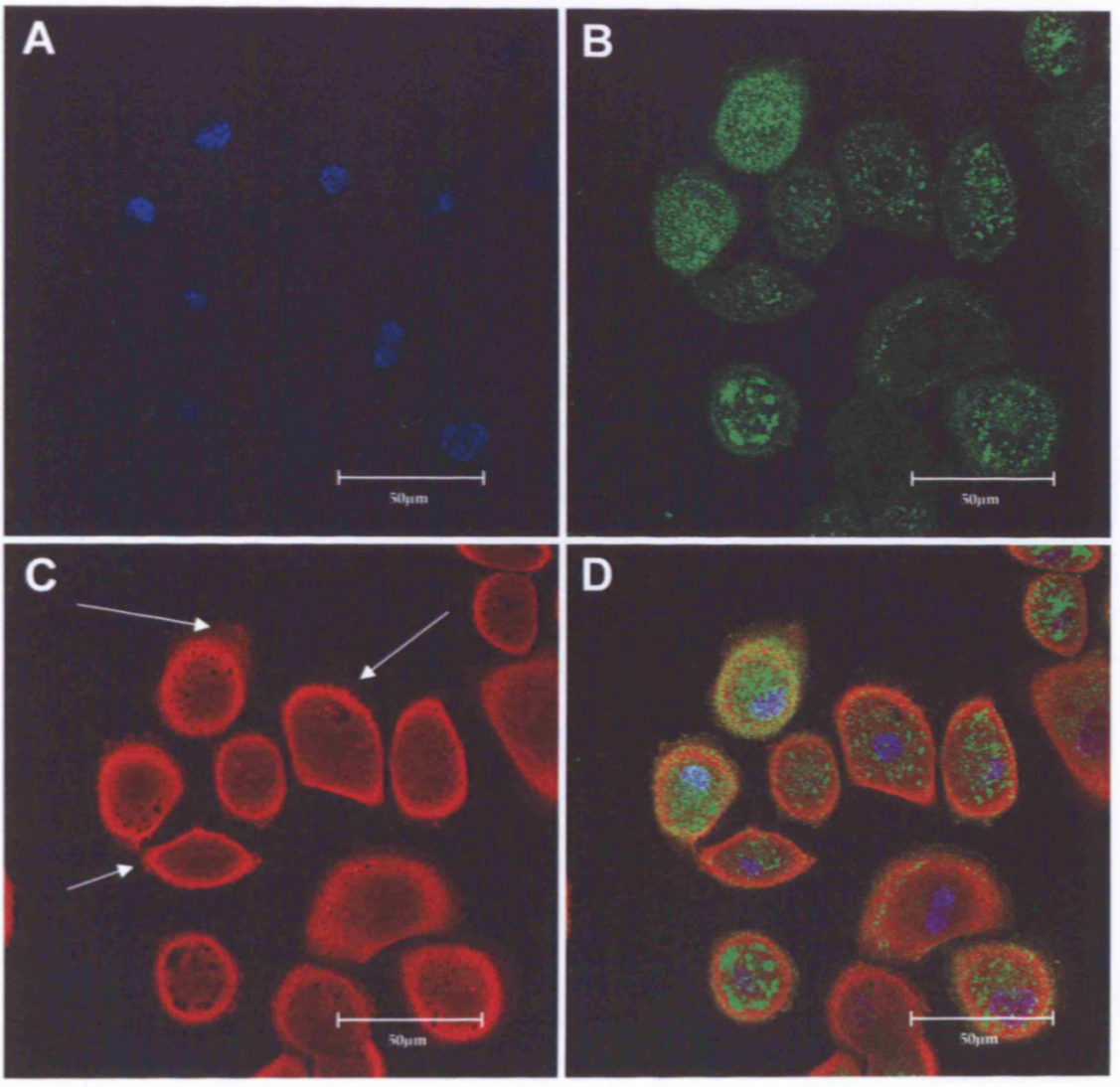


Figure 4.11 Staining of the nuclei (A), αvβ6 integrin (B), actin cytoskeleton (C) and a composite of the three (D) of VB6 plated on the 120kDa FN fragment. Lamellopodia and filipodia are apparent (arrows).

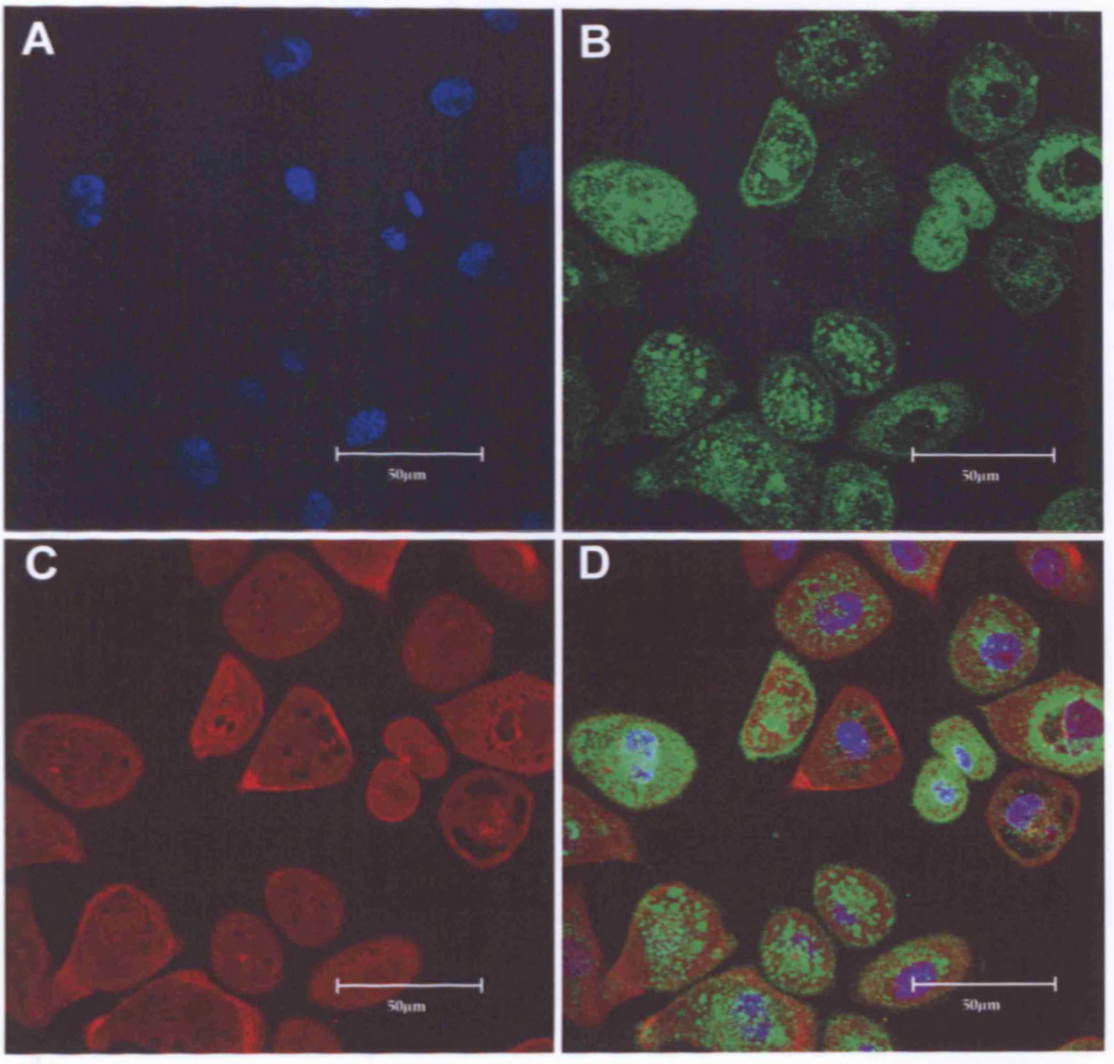


Figure 4.12 Staining of the nuclei (A), αvβ6 integrin (B), actin cytoskeleton (C) and a composite of the three (D) of VB6 plated on pFN.

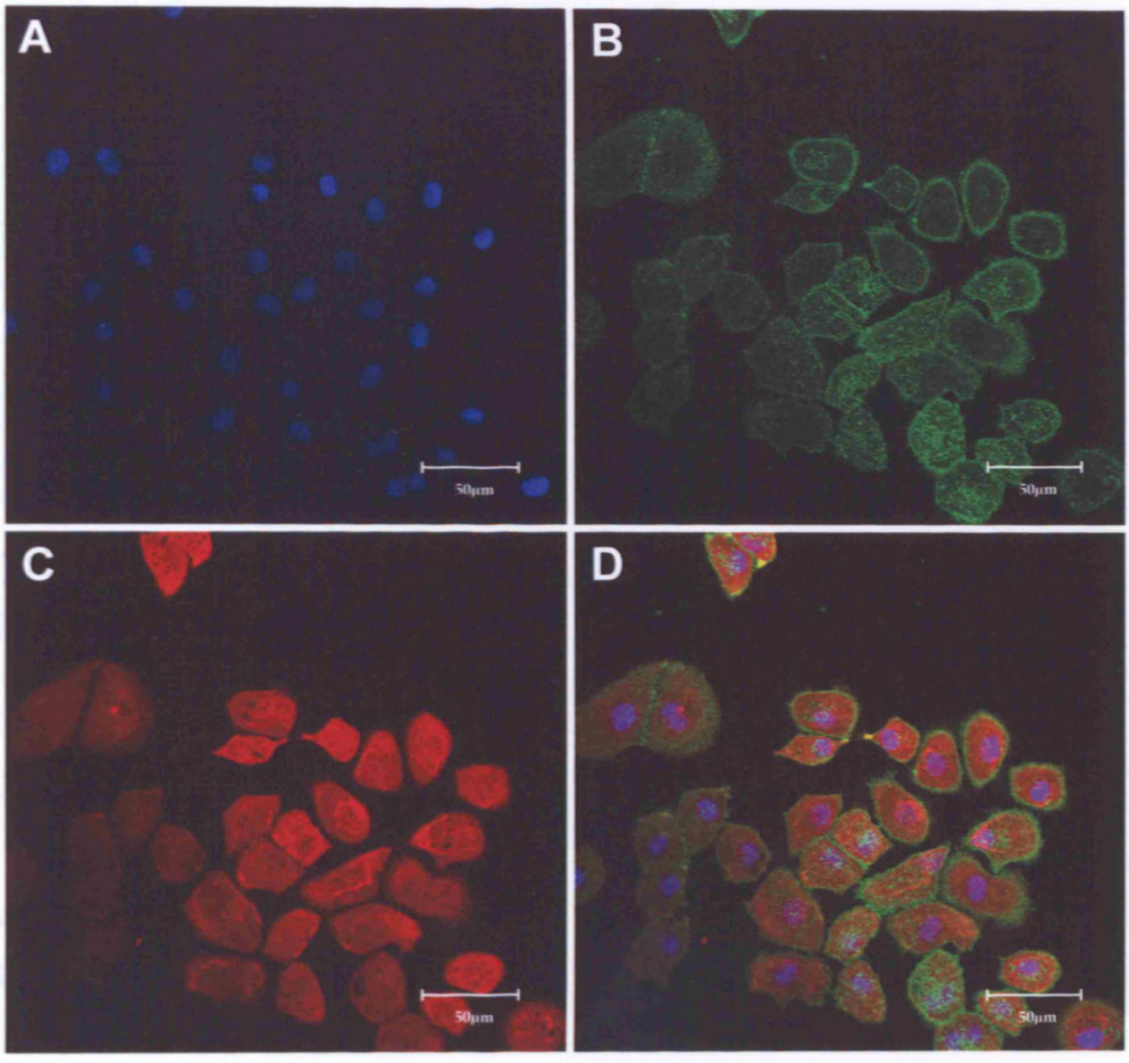


Figure 4.13 Staining of the nuclei (A), E-cadherin (B), actin cytoskeleton (C) and a composite of the three (D) of VB6 plated on 120kDa FN fragment.

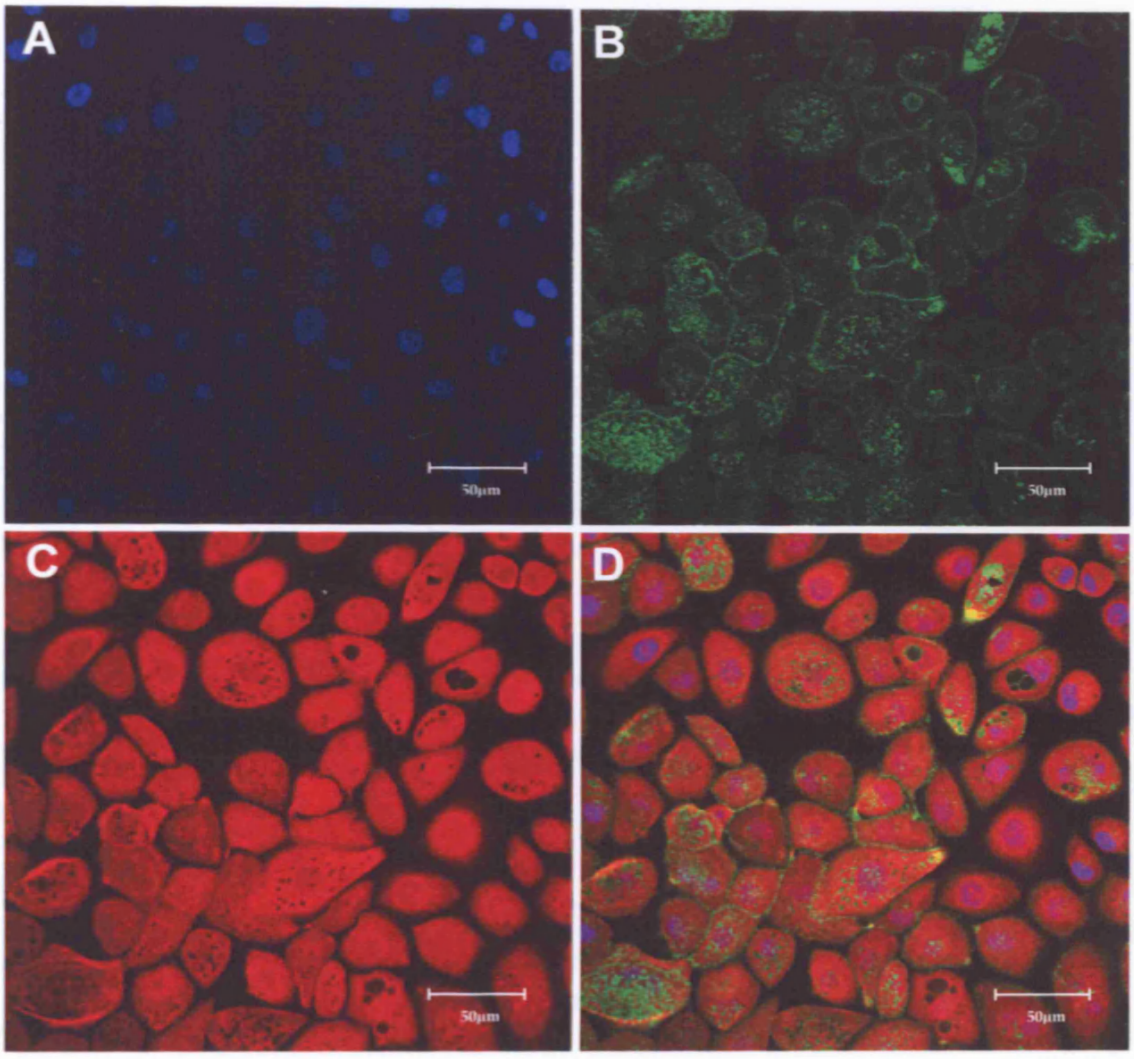


Figure 4.14 Staining of the nuclei (A), E-cadherin (B), actin cytoskeleton (C) and a composite of the three (D) of VB6 plated on pFN.

4.5 DISCUSSION

Paradoxically, the feature of epithelial cells that enables wounds to heal is also the one that facilitates the spread of cancer: enhanced motility. Tight regulation of such motility is what differentiates the two conditions from each other and renders one "benign" and one "malignant". During wound healing, keratinocytes become activated and start migrating into the wound. Such migration involves the coordinated expression of extracellular matrix proteins, their receptors and proteinases (Larjava *et al.* 2002). When there is impairment of the regulatory process and loss of its coordination, tumourigenesis may occur. Whether in wound healing or in tumour invasion and metastasis, the result is due to cell responses to microenvironmental motility cues (Quaranta 2002). Migrating keratinocytes change morphology from a polarized basal keratinocyte to an elongated and flattened one with cytoplasmic processes and ruffled edges, resembling a mesenchymal phenotype (Mercurio & Rabinovitz 2001; Larjava *et al.* 2002). An additional characteristic is a change in their expression pattern of integrins, namely $\alpha5\beta1$ and $\alpha v\beta6$. These integrins are typically expressed at low levels in normal, healthy, adult epithelium (Haapasalmi *et al.* 1996; Hakkinen *et al.* 2000; Koivisto *et al.* 2000). While expression of the $\alpha5\beta1$ integrin is high during initial migration, the $\alpha v\beta6$ integrin expression is maximal when migration is well established (Haapasalmi *et al.* 1996).

Proteolytic fragments of ECM have a wide range of activities including chemoattraction. Among the various effects such fragments have been found to exhibit are: enhanced dermal fibroblast chemotaxis, keratinocytes migration and induction of human platelet aggregation (Pilcher *et al.* 1997; Messent *et al.* 1998; Stringa *et al.* 2000). FN, collagen IV and laminin are powerful simulators of migration of tumour cells and non-transformed cells. Studies on extracellular fragments have already been carried out on several extracellular molecules. Degradation products of collagens are involved in a variety of processes such as endothelial and blood cell chemotaxis (Ortega & Werb 2002), chondrocyte MMP secretion and downregulation of collagen secretion (Messent *et al.* 1998), anti-angiogenesis and migration of neural and non-neural cells (Ortega & Werb 2002). Break-down products of collagen IV with exposed cryptic sites have been shown to promote cell migration in carcinoma cells (Xu *et al.* 2001). Laminin fragments have been implicated in remodelling mouse tissue (Koshikawa *et al.* 2000), rodent skin carcinoma (Koshikawa *et al.* 2000) and remodelling mammary glands (Koshikawa *et al.* 2000). Cleavage of laminin-5 by MMPs has been observed to increase motility markedly in carcinoma cells (Giannelli *et al.* 1997).

Migrating keratinocytes express alternatively spliced EDA FN whose RGD sequence is recognized by both $\alpha5\beta1$ and $\alpha v\beta6$ integrins (Larjava *et al.* 2002) and it is thought that FN may participate in the induction of its own receptor (Larouche *et al.* 2000) . Studies have established the pivotal role played by FN

in both wound healing and tumourigenesis and the ability to migrate over FN may be a crucial step in tumour invasion and metastasis (Koivisto *et al.* 2000). On account of the modular nature of the information provided to the cell by the different domains of FN, different proteolytic fragments evoke cellular responses that are distinctly different from those provoked by the intact molecule. Cell-binding fragments, and not heparin- or collagen/gelatin-binding domain of FN or intact FN, modulate the expression of proteinases (Hu *et al.* 2000). FN fragments induce proMMPs, uPA and decrease TIMP-1 and TIMP-2 (Hu *et al.* 2000). Some have suggested that the distinct differences in cellular response to the 120 kDa FN fragment and the intact molecule may reside in the IIICS site of FN (Hu *et al.* 2000).

4.5.1 Fragments and migration

The migration assays that were carried out measure the fragments' chemotactic properties by a concentration gradient. An apparent increase in migration of β6 expressing cells towards the 120kDa cell-binding FN fragment was observed. Binding to this fragment is through the αvβ6 integrin by recognition of the RGD sequence. It also binds the α5β1 integrin and in this case the PHSRN sequence acts as a synergistic site. Our results show not only greater migratory stimulation by the cell-binding fragment, but also found the VB6 cells to be more migratory compared to the β6 null transfectant parent cells, C1. This confirms previous experiments performed in our laboratories (Thomas *et al.* 2001a). The more malignant phenotype associated with the

expression of the β6 integrin has been shown by others to be over-expressed in colon carcinoma cells and involved in the promotion of tumourogenicity (Varner & Cheresh 1996; Agrez *et al.* 1999). Our results not only depict the cell-binding fragment as an active mediator of migration but also establish the important role played by the αvβ6 integrin. Other groups have observed that enhanced expression of various extracellular matrix proteins was found maximal underneath keratinocytes expressing this integrin (Clark *et al.* 1988; Clark *et al.* 1996; Haapasalmi *et al.* 1996). Although Liu *et al.* (1998) have shown the heparin-binding 30kDa FN fragment to be important in increased migration of cells in soft tissue sarcomas, our experiments showed no effect with this fragment nor with the peptide. This suggests the presence of other sites within the 120kDa FN fragment, not present in the other two fragments, which are responsible for the increased migration. Furthermore, the αvβ6 integrin doesn't bind to either the 30kDa FN fragment or the PHSRN peptide.

Cultures of SCC cells have shown that the deposited FN matrix was made up of truncated and less organized fibres and the addition of anti-αvβ6 antibody restored the deposition to an organized FN matrix (Ramos *et al.* 2002). Liu *et al.* (1998) have shown that the conditioned medium from oral squamous carcinoma cells is rich in promigratory FN fragments. Kapila *et al.* (1996) have shown that the 120 kDa FN fragment possesses activities not found in the parent molecule which affects proliferation, and stimulates migration. Active proteolysis of FN may occur close to cells undergoing neoplastic

transformation. Such fragments can induce the generation of plasmin by cancer cells and can lead to the activation of MMPs which not only break down extra cellular matrix barriers, but also degrade FN further and release pro-migratory fragments thus forming a loop (Grant *et al.* 1998).

Our wound assay experiments showed no difference in migration between the fragment and the intact molecule. This could be on account of the different environment of the wound assay. In this assay the cells were either seeded on the matrix protein, or incubated with it, and left overnight. Cells produce their own matrix proteins and therefore receive signals in a different manner from the gradient of migration assays. In migration assays we are seeing the isolated effect of the fragment's exposed cryptic sites on the cells without the involvement of any other factor. Moreover, when keratinocytes migrate laterally from a cell cluster migration seems to be mediated mainly by the β1 integrins rather than the β6 integrins (Larjava *et al.* 2002). Our findings suggest that β6 integrins are active in the presence of migration gradients but play a lesser role in more complex environment when more that one ECM molecule may be present. Cultured keratinocytes and keratinocytes in vivo during wound healing show the ability to switch from one integrin to another indicating a level of integrin redundancy (Larjava *et al.* 2002).

Measurement of migration of NHK on the same matrix proteins showed increased migration towards the intact molecule or just lower migration

towards the fragment. Normal human keratinocytes express lower levels of the αvβ6 integrin than their malignant counterpart and activated keratinocytes in wound healing (Thomas *et al.* 2001b) and are generally less migratory expressing higher levels of E-cadherin. Growth in culture stimulates re-expression of the αvβ6 integrin, however, it is still considerably less than the VB6 cells (Figure 3.7). It is reasonable to conclude that the αvβ6 integrin may be pivotal to the cells' response to the fragment.

4.5.2 The αvβ6 integrin and migration

The α5β1 integrin is seen as a prototype FN receptor since it is specialized for binding to this ligand and is expressed by many cell types for which it is the major FN receptor present. The RGD loop in III_{10} is the critical recognition site for α5β1 but the synergy site PHSRN is required for high affinity binding (Livant *et al.* 2000). The αvβ6 integrin is expressed by epithelial cells during development and wound healing and by many tumour cells. Agrez *et al.* (1994) have shown that the re-expression of the αvβ6 integrin has been implicated to increase proliferation of epithelial cells. However, this effect was shown to be absent when plating the cells on matrix proteins such as collagen or FN. The effect was nonetheless pronounced in three-dimensional culture environments. This fits with the observed increased migration of β6 expressing cells on FN fragment and suggests a more motile phenotype. Fibronectin is αvβ6 integrin's main ligand to which it also binds via the RGD sequence, but does not require the synergy site. It is the only integrin

expressed exclusively by epithelial cells (Johansson *et al.* 1997). This *de novo* expression suggests that its presence may modify the cells phenotypic activities. Others have shown that increased expression of this integrin was found to be the main individual mediator of cell movement in migration assays (Huang *et al.* 1998).

Blocking experiments were carried out to determine the importance of each integrin in this migration. Groups working on breast tumour cells showed that anti-α5 antibodies reduced FN-induced migratory properties and FN-mediated adhesion (Bartsch *et al.* 2003). Others have shown that blocking the α5β1 integrin by the addition of a function-blocking antibody (P1D6) greatly reduced fragment-mediated migration in keratinocytes and fibroblasts (Livant *et al.* 2000). This is consistent with our findings. Migration of VB6 was reduced when both integrins were blocked separately; however, maximal inhibition of migration was shown when both integrins were blocked, suggesting an additive effect.

4.5.3 Fragments, adhesion and proliferation

Inhibition of tumour cell adhesion is not always associated with reduced migration and this can be explained by adhesion being primarily related to the binding of integrins to ECM whereas migration is more complex and involves cycles of adhesion and de-adhesion as well as the activation of intracellular signalling pathways which in turn lead to varying cellular

activities. Many have shown that no single integrin antagonist could totally abolish breast cancer cell adhesion and migration on FN, and it can be concluded that more than one integrin is involved in the process. Malignant tumours express great degrees of heterogeneity in integrin expression in breast cancer cells (Bartsch *et al.* 2003).

Having establishing the pro-migratory properties of the cell-binding fragment, proliferation and adhesion assays were carried out. The aim was to ascertain whether the increase in migration was a result of an increase in the cells' proliferative activities. Others have shown that the 120kDa FN fragment reduces proliferative activity and enhances apoptosis in mammary epithelial cells (Schedin *et al.* 2000). Our assays showed no increase in proliferation of VB6 plated on fragment or whole molecule and this is inconsistent with findings of others (Agrez *et al.* 1994). Therefore it seems that these fragments exhibit their effect via β6 integrin alone and are independent of alterations in cell growth and proliferation.

The migration process involves the recognition and attachment of the cell to ECM molecules, followed by the detachment of the trailing edge and bodily translocation in the direction of the chemotactic stimuli (Mercurio & Rabinovitz 2001). Having established that cells in our studies recognized the cell-binding FN fragment via their integrin receptors, adhesion assays were carried out to establish whether the increased migration was dependent on

any changes in adhesion. Results showed no difference between the matrix molecules tested, indicating that, rather than decreasing the adhesion (with subsequent increase in migration), the fragment acts as a signal, independent of a quantitative change in attachment. It seems that the role of the cell-binding fragment is in the stimulation of single cell migration via the αvβ6 integrin (as opposed to the α5β1 integrin). The αvβ6 integrin has been implicated in this migration by several studies (Larjava *et al.* 2002) and has been shown to play an important role in the migration studies described in this thesis.

4.5.4 Migration and morphology

When a keratinocyte changes from a stationary cell to a migratory one, its morphology changes accordingly. Among the changes is the appearance of lamellipodia and filipodia (Kassis *et al.* 2001; Mercurio & Rabinovitz 2001). One of the most important functions of FN is maintenance of cell morphology via organization of cell attachment to ECM. After plating VB6 on various matrices, the cells exhibited varying degrees of spreading and morphologies. A more migratory phenotype was seen in cells plated on the fragment as compared to the intact molecule. However, no evidence of a down-regulation of E-cadherin in cells plated on the fragment was observed. There exists an inverse relationship between the expression of E-cadherin and the migratory potential of the cell (Wheelock & Johnson 2003). We could assume a reverse relationship between the β6 integrin and E-cadherin, and the fragment may

possibly influence the cells to have a more active β6 integrin and a more quiescent E-cadherin. Although others have shown a down-regulated expression of this cell-adhesion molecule in invasive cancer cells (Margulis *et al.* 2003) there was no evidence of a difference in E-cadherin expression of cells plated on plasma FN and the 120 kDa FN fragment. The reasons for our findings could be that staining experiments, while detecting the presence of the antigen, do not give any information on the activity of the molecule.

4.5.5 Conclusion

It seems likely that the intact FN molecule, found in copious amounts in active wound healing and tumourigenesis, is cleaved by cellular proteases to yield fragments that possess different properties from the parent molecule. In this work we studied both the heparin-binding domain 30 kDa FN fragment and the 120kDa cell-binding fragment and found the latter to upregulate migration of β6 expressing cells. This increase in locomotion is not accompanied by increased proliferation or adhesion, and is blocked by anti-β6 antibodies. We have also shown that the α5β1 integrin plays a role but believe it to be secondary. Morphologically, cells exhibit a migratory phenotype when plated on the fragment.

In theory, blocking the action of this FN fragment should inhibit or block invasion. Bartsch *et al.* (2003) synthesized an antagonist peptide to block the binding of α5β1 integrin and consequently block or alter the effects of ligand

binding. Although this was successful in vitro, cancer cells have shown a cunning ability to adapt and utilise other integrins to bypass such blockades. This reaffirms the complexity of the mechanisms involved and indicates that other molecules such as MMPs and proteases may also play a role in this increased migration.

CHAPTER 5

FIBRONECTIN FRAGMENTS AND PROTEASE SECRETION

5.1 INTRODUCTION

Tumour invasion and metastasis depend on the ability of the tumour cells to co-ordinate a series of biological events implicated in the formation and degradation of structural matrix proteins (Reuning *et al.* 1998). Tumour invasion and wound healing are both processes that involve the active migration of cells. Cell migration into the surrounding ECM is inherently linked to a localized cell surface proteolytic activity which favours the cell detachment from the ECM and invasion into the surrounding degraded matrix. This mechanism is brought about by the interaction of integrins and proteases, namely uPA and MMPs. Tumourigenesis differs from normal wound healing in that cancer cells use uPA or MMPs in conjunction with some integrins at times or places that is incompatible with normal cell behaviour (Clezardin 1998). Upregulation of the αvβ6 integrin in SCC suggests a role in tumour progression and possible role in the upregulation of such proteases (Thomas *et al.* 2001a).

5.1.1 Fibronectin and MMPs

Matrix metalloproteinases play an important role in tumour growth and metastasis by degrading matrix barriers and by enhancing angiogenesis (Westermarck & Kahari 1999). Furthermore they can release growth factors

and angiogenic factors from the cell surface and ECM (Suzuki *et al.* 1997). A great deal of evidence implicates MMPs in cancer spread (Westermarck & Kahari 1999) and it seems that the interaction occurring between integrins and MMPs is the general mechanism commonly employed during tissue remodelling and tumourigenesis (Clezardin 1998).

5.2 OBJECTIVES

To assess the effect of the FN fragments on the production of the serine protease uPA and the effect of the 120kDa FN fragment on matrix metalloproteinases MMP-2 and -9 production in both β-6 expressing cell line (VB6) and the null transfectant (C1).

5.3 MATERIALS AND METHODS

Protease expression can be measured by different techniques that assess the production of proteases either directly or indirectly. They either measure the actual presence of the proteins (Western Blots) or measure their activities (plasminogen activator assays and zymography).

5.3.1 Plasminogen Activator Assay

Six-well plates (Nunc™, VWR International) were coated with pFN, 120kDa FN fragment, 30kDa FN fragment and PHSRN peptide in PBS at a concentration of 1μM/ml. Cells were seeded at a concentration of $3x10^5$ in defined SFM and left overnight. The following day the supernatant was collected and assayed by plasminogen activator assay as described in section 2.3.1. In another set of experiments the matrix proteins were added to the medium (in solution) at the same concentrations, rather than coating.

5.3.2 Zymography

Cells were grown on wells coated with pFN, 120FN fragment in PBS and PBS alone at a concentration of 1μM/ml overnight in Defined Keratinocyte medium at a seeding density of 10^5. The following day the supernatant was collected, centrifuged to remove cells and debris and loaded with non-reducing sample buffer (Appendix 1) into a 10% SDS PAGE containing gelatin and electrophoresed for 1 hour (see section 2.3.3). Following electrophoresis

the gels were washed and placed in Developing Buffer overnight (see section 2.3.3) The following day the gels were stained with Commassie blue, destained and images were taken with Alpha imager 1220 version 5.5 (Alpha Innotech Corp™, USA). The data was then analyzed using Scion Image (Scion Corporation).

5.4 RESULTS

5.4.1 The 120kDa FN fragment does not influence the production of uPA

Experiments were set up in order to assess the effect of the various FN fragments on the secretion of proteases. The fragments used include the 30kDa amino-terminal fragment and the 120kDa cell-binding fragment. The first set of experiments checked for the uPA levels. We observed that the αvβ6 expressing cell line, VB6, produced more uPA than their negative counterpart, C1. Two sets of experiments were run in duplicate examining the effects of the fragments, one by coating the fragments in the wells and then seeding the cells therein, and the other by seeding the cells in uncoated wells and adding the fragments in solution, i.e. in the medium. Our data shows that both the 30kDa and the 120kDa FN fragment did not upregulate the production of uPA in either cell line whether seeded on fragment coated wells or with the fragments added to the medium (Figure 5.1, 5.2, 5.3 and 5.4). The PHSRN peptide showed similar results (Appendix 2). There was however, a difference between VB6 and C1 and this was seen consistently throughout the experiments.

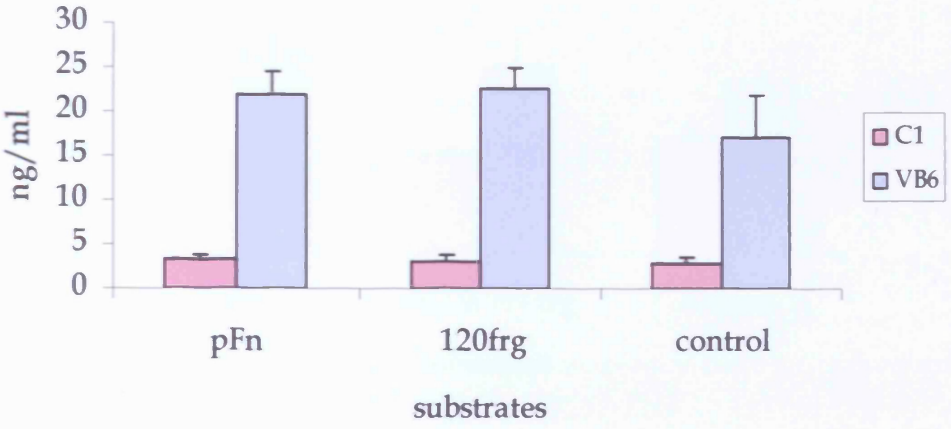


Figure 5.1 PAA of cells seeded on wells coated with pFN, 120kDa FN fragment and PBS. There is no difference in uPA production between pFN, the 120 kDa FN fragment and the control in both cell lines. The difference observed here is in the higher baseline production of uPA in VB6 compared to C1.

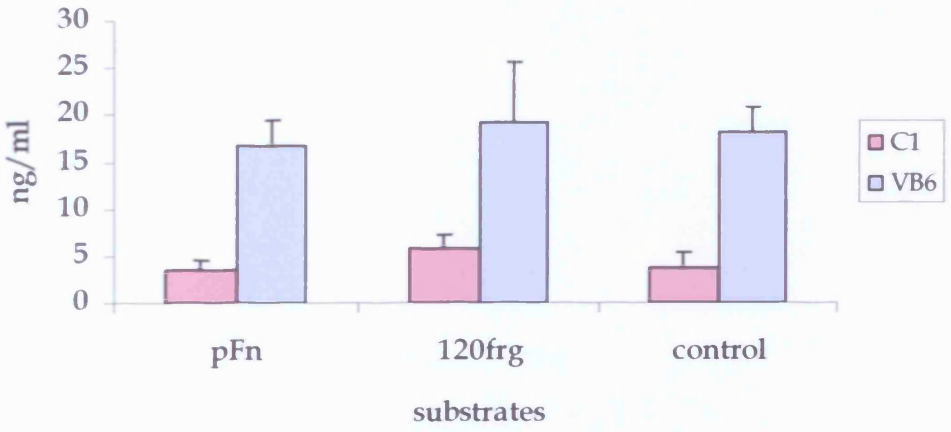


Figure 5.2 PAA assay of cells incubated with pFN, 120kDa FN fragment and PBS. Similarly, no observed difference in uPA production in each cell line plated on the various matrices, but again, higher baseline production in VB6 cells.

5.4.2 The 120kDa FN fragment up-regulates the expression of both pro-MMP-2 and pro-MMP-9 in αvβ6 expressing cell lines

Zymograms were carried out using supernatants from NHK, C1 and VB6 seeded on 24 well plates coated with pFN, 120kDa FN fragment and PBS. Our results showed an increase in pro-MMP-2 production in both cancer cell lines, but a more pronounced difference between the pFN and the 120kDa FN fragment in the β6 expressing cell lines (Figure 5.5, 5.6, 5.7 and 5.8). The normal human keratinocytes exhibited no difference in production when plated on the various substrates (Figure 5.9 and 5.10).

Since the αvβ6 integrin has been implicated in the upregulation of MMPs (Thomas *et al.* 2001a), we ran experiments with β6-blocking antibodies to check if blocking this integrin would down-regulate the production of the MMPs. Our results showed no difference between the cells with and the cells without the anti-β6 antibody (Figure 5.11 and 5.12).

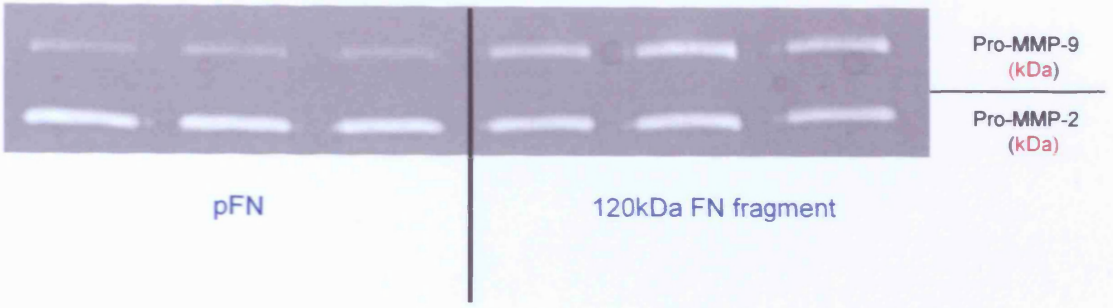


Figure 5.5 Gelatin lysis bands on Zymogram gel of VB6 plated on pFN and 120kDa FN fragment.

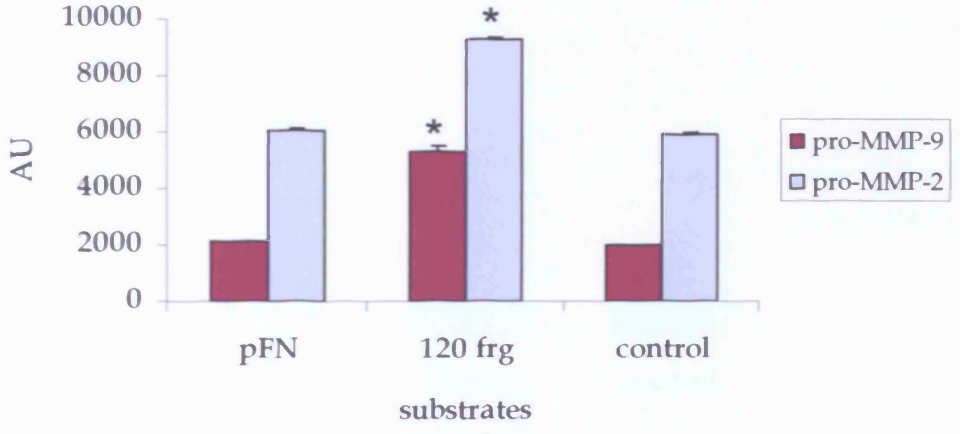


Figure 5.6 Graphic representation of Zymogram carried out on the supernatants of VB6 exposed to wells coated with pFN, 120kDa FN fragment and PBS. Both Pro-MMP-2 and pro-MMP-9 are significantly upregulated when plated on the 120kDa FN fragment (p value= 0.001).

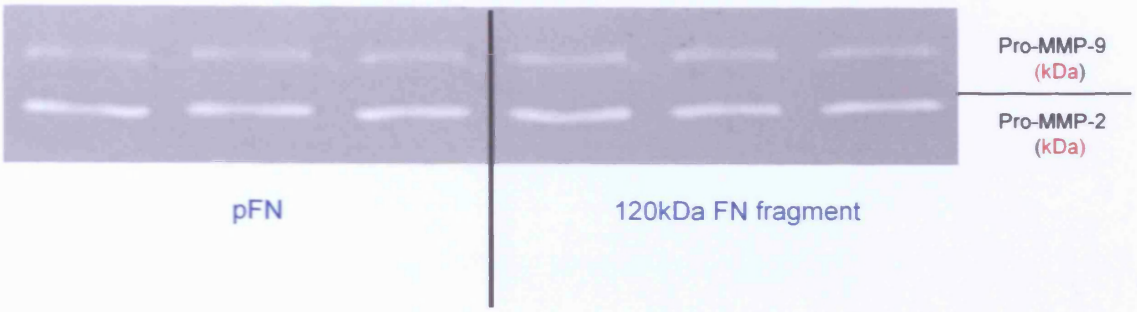


Figure 5.7 Gelatin lysis bands on Zymogram gel of C1 plated on pFN and 120kDa FN fragment.Figure

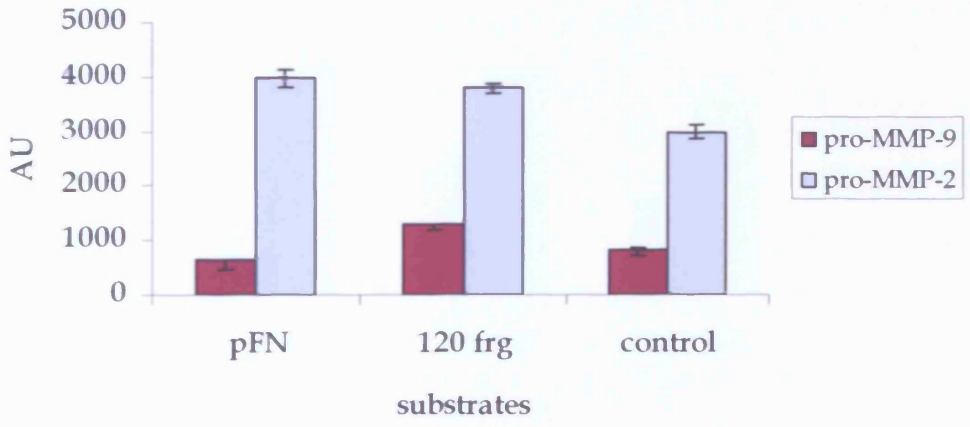


Figure 5.8 Graphic representation of Zymogram carried out on the supernatants of C1 exposed to wells coated with pFN, 120kDa FN fragment and PBS.

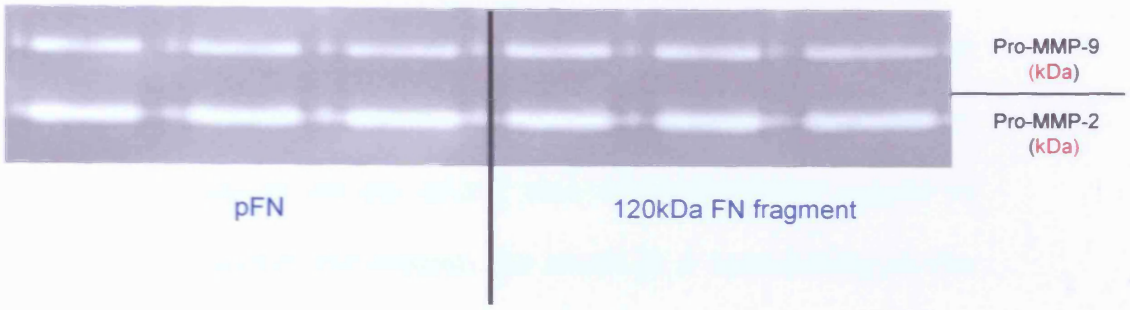


Figure 5.9 Gelatin lysis bands on Zymogram gel of NHK plated on pFN and 120kDa FN fragment.

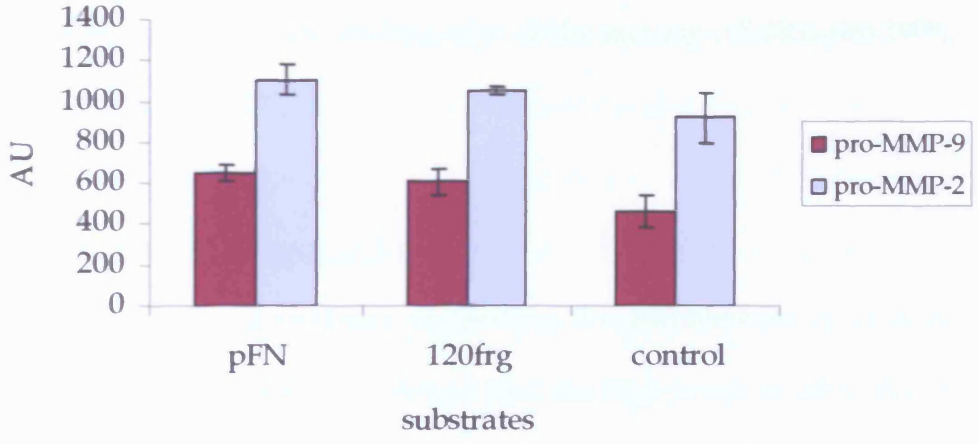


Figure 5.10 Graphic representation of Zymogram carried out on the supernatants of NHK exposed to wells coated with pFN, 120kDa FN fragment and PBS.

5.5 DISCUSSION

When discussing cell motility, one envisions a pathway through which the cells move. This pathway is created by the cells to either encourage the closure of a wound, or as an erratic and self-propagating means in tumourigenesis. Whatever the reason, the result is a remodelling of the extracellular matrix that renders it more penetrable and that presents novel molecules, or fragments of original molecules, that enhance and encourage further motility. It has been observed by many that a cell not only changes in morphology when it receives motility cues, but also starts to secrete proteases and rearrange its cell-adhesion molecules and receptors to adapt to the new surroundings. In migrating keratinocytes particularly, upregulated protease secretion seems to occur on the leading edge of the moving cell (Shapiro 1998; Murphy & Gavrilovic 1999).

5.5.1 The 120kDa FN fragment and uPA

There is a large body of evidence suggesting the involvement of uPA in cancer metastasis. It has been established that the high levels of uPA, PAI-1 and uPAR correlate with poor prognosis and the level of uPA was found to regulate the rate of cell migration (Andreasen *et al.* 1997; Decock *et al.* 2005). PAI-1 seems to promote cancer formation by regulating cell adhesion and migration (Sakakibara *et al.* 2005). Such motility is inhibited by antibodies against uPA (Andreasen *et al.* 1997). uPA is able to digest FN independently

of plasmin and also activates other matrix degrading enzymes such as MMP-2 and MMP-9 (Reuning *et al.* 1998). Proteolysis of FN by uPA yields two large polypeptide chains, and this digestion seems to be efficient at a relatively high enzyme: substrate ratio, as opposed to plasmin. It could be possible that uPA causes an initial cleavage of FN reducing it to its monomeric form and this form is in turn more susceptible to further degradation by other proteolytic enzymes (Gold *et al.* 1992).

Although the focus of our study was on two families of proteases, the urokinase plasminogen activator serine protease, and the matrix metalloproteinases, MMP-2 and MMP-9, we started by running several assays to assess the production of uPA by both, beta-6 transfected cells, VB6 and the null transfectant, C1. UPa (and its receptor uPAR) plays an important role in the tissue processes of localized and directional proteolysis, and has been found to stimulate the migration and proliferation of a variety of tumour and normal cells (De *et al.* 2002). High levels of uPA mRNA has been found to be indicative of poor prognosis (Gold *et al.* 1992; De *et al.* 2002). Our assays failed to show an upregulation of uPA when the cells were plated on the 120kDa FN fragment as compared to pFN, but did confirm previous data showing elevated secretion of uPA by beta-6 expressing cell lines (Kapila *et al.* 1996; Hu *et al.* 2000; Ahmed *et al.* 2002b). This fits the phenotype of VB6 as they are more aggressive and more migratory on account of their expression of the $\alpha v\beta 6$ integrin (Thomas *et al.* 2001b; Ramos *et al.* 2002). The same experiment

was carried out incubating pFN and the 120kDa FN fragment in the medium rather than coating on the wells. It is possible that suspension in medium exposes cryptic sites that are hidden when the fragment is attached to a surface. When FN is adsorbed to surfaces it undergoes conformational changes that could affect its activity (Hynes 1999). Since the nature of FN is elastic, FN exists in the pericellular matrix in a stretched form which could lead to differences in integrin receptor binding (Garcia *et al.* 1999). Work by Hu *et al.* (2000) showed an increased production of uPA by fibrocartilagenous cells when exposed to the 120kDa FN fragment. Upregulation of uPA, collagenases and stromelysin was observed in periodontal ligament cells in response to the cell-binding fragment (Kapila *et al.* 1996). However, uPA assays failed to show differences in uPA production regardless whether the cells were seeded on the substrates or incubated with them. This was also the case for the 30kDa FN fragment and the PHSRN synergy peptide. Another reason could be that the cells were already producing uPA in maximal amounts and plating or incubating with fragments was therefore not inducing any difference.

5.5.2 The 120kDa FN fragment and MMPs

Both MMP-2 and MMP-9 have been investigated thoroughly in cancers. MMP-2, for example, has been implicated as a marker for malignant transformation of cervical epithelial cells (Davidson *et al.* 1999; Talvensaari-Mattila *et al.* 1999). MMPs are mainly secreted by non-malignant stromal cells

in malignant tumours. Integrins induce the expression of different ECM degrading proteases especially MMPs and the interplay between them could be one of the key phenomena in the invasion process (Koistinen & Heino 2002). For example, integrin αvβ6 has been shown to increase the expression of MMP-1, MMP-3 and MMP-9 (Werb *et al.* 1989). It has been suggested that FN fragments may be inducers of MMP activity and mediators of tissue remodelling in wound healing (Schedin *et al.* 2000).

We assessed MMP activity by the same cell lines, again running a comparison between the 120kDa FN fragment and the intact pFN. We found an upregulation of pro-MMP-2 and -9 by the VB6 which may at least in part explain the increase in migration noted in previous experiments (see chapter 4). Integrins have been found to induce the expression of different ECM degrading proteases, especially members of the MMP family. Studies by Schedin *et al.* (2000) have shown that mammary epithelial cells upregulated their expression of MMPs when exposed to FN and its fragments. It has been reported that the induction of MMP peaks with the peak in FN fragmentation, and that the use of an MMP inhibitor suppressed this finding (Schedin *et al.* 2000). The 120kDa FN fragment has been shown to induce expression of pro-MMP1 and pro-MMP-3 in cynoviocytes but not the intact molecule (Werb *et al.* 1989). In fibroblasts, signalling by FN and its fragment occur via the α5β1 integrin and adding functional blocking antibodies to this integrin reduced dramatically the MMP-9 induction by the fragment (Schedin *et al.* 2000). Our

study shows that this effect is further synergized by the αvβ6 integrin since both VB6 and C1 exhibit similar expression patterns of α5β1. The production of pro-MMP-9 might be already at maximal levels in both cell lines plated on the cell-binding fragment, but the presence of the αvβ6 integrin further upregulates it. This finding is shared by others. Esparza *et al.* (1999) has shown that lymphocytes exposed to FN produced increased amounts of MMP-2 and MMP-9 over time. Werb *et al.* (1989) have found that FN increases the expression of MMP-1, MMP-3 and MMP-9 in rabbit synoviocytes via the α5β1 integrin. Kapila *et al.* (1996) have shown the effect of intact and the 120kDa FN fragment on periodontal cells. Both ligands are able to stimulate the expression of different proteases. In the case of the 120kDa FN fragment not only were MMPs upregulated but also uPA which may be responsible for the activation of several MMPs. In colon cancer, the aggressiveness of the tumour has been linked to its expression of MMP-9 and TIMP-1. Again, the α5β1 integrin has been shown to induce collagenases and stromelysin expression. Since the αvβ6 integrin is expressed de novo and has been found to be concentrated at the leading edge, it has been postulated that it plays a role in the upregulation of proteases since that is where proteolysis takes place. In colon cancer cell lines, like in our transfected keratinocytes, there is an increase in expression of MMP-9 in beta-6 expressing cells as opposed to null transfectant (Agrez *et al.* 1999).

5.5.3 MMP-uPA-stroma cross-talk

Despite identifying MMP-2 and MMP-9 as the upregulated proteases in the presence of the 120kDa FN fragment, this does not exclude the involvement of uPA from in the increased motility seen in our work. Studies by many have provided us with the basic information that uPA is capable of cleaving FN near the C-terminus proximal to the disulfide bridges linking the two monomers with the release of two 230-250kDa FN fragments (Gold *et al.* 1992; De *et al.* 2002). Therefore, not only is uPA capable of stimulating the expression of FN, but it can also then cleave the secreted glycoprotein (De *et al.* 2002). The cleaved FN is then prone to further breakdown (Gold *et al.* 1992). Such degradation and release of FN occurs at the cell-substratum contact sites, the same place where cell-bound uPA has been localized. It seems that such a location for uPA concentrates the enzyme at the surface of proteolytically active cells although a study by Dalvi *et al.* (2004) has found the αvβ6 expressing cells downregulate the expression of uPA receptor uPAR. UPa not only activates plasminogen into plasmin, responsible for the degradation of the ECM, but it also independently cleaves FN into more degradation-susceptible fragments that may possess innate biologic activities (Gold *et al.* 1992). This has provided part of the loop we observe in tumourigenesis and wound healing. For example, it has been estimated that up to 50% of the FN in arthritic synovial fluids has been proteolytically fragmented. FN fragments, including the collagen-binding domain and the cell-binding domain have been shown to promote loss of cartilage

proteoglycans in vitro. It is reported that the FN fragment induced cartilage degradation is due to increases in metalloproteinases expression and activity (Huhtala *et al.* 1995). Fibronectin fragments exhibiting the RGD integrin-binding site regulate the synthesis of MMPs via an Interleukin-1 autocrine loop. MMPs then act on FN to release FN fragments (Stanton *et al.* 2002). This could be seen as part two of the loop (Figure 7.1)

Cellular invasion of the surrounding matrix involves the interaction of different proteases; the MMPs appear to be involved in the initial step in this process. In many tumours there is varied expression of MMPs both in tumour cells and in the surrounding reactive tumour stroma (Davidson *et al.* 1999; Talvensaari-Mattila *et al.*1999; Reunanen & Kähäri 2002). In many cases the level of expression of different MMPs has been correlated with tumour grading and clinical staging. However, no overall pattern of MMP expression in different types of human cancers has been developed (Stetler-Stevenson & Yu 2001). In order for MMP proteolysis not to lead to widespread destruction of the ECM, activation is closely regulated and maintained next to the cell surface. A consequence of such ECM remodelling is that changes within this three-dimensional structure may reveal cryptic sites in its components (FN being one of the more important ones) previously unrecognizable by the cell-surface receptors (Streuli 1999). A variety of MMPs are expressed both during normal physiological processes and during tumourigenesis, and this is perhaps to compensate for potential loss of an individual MMP. Such

diversity explains why MMP-mutant mice are only mildly affected with respect to development and other physiological processes (Shapiro 1998).

5.5.4 Conclusion

There is continuous talk between cancer cells, stromal cells and inflammatory cells during invasion. Our results show an upregulation of MMP-2 and MMP-9 by β6 expressing cells when plated on the 120kDa FN fragment, which suggests their involvement in the increased migration observed in chapter 4. The upregulated MMPs are in their zymogen states, and in fact, most MMPs are secreted as zymogens and are proteolytically activated in the ECM space. Plasmin and stromelysin are possible candidates for the activation of pro-MMPs into active enzymes. Beta-6 expressing cell lines and not the null transfectant produce more uPA and this is responsible for the activation of plasmin from plasminogen. This in turn could be responsible for the activation of MMPs. It was found that recombinant PAI-2 and aprotonin (anti-plasmin) inhibited the enhanced degradation of plasmin by beta-6 transfectants (Agrez *et al.* 1994). This could be the link tying the observed increased production of uPA by VB6 and their then upregulated expression of pro-MMP-2 and -9. This argument is tackled in further depth in the following chapter.

CHAPTER 6

ROLE OF MATRIX METALLOPROTEINASES IN FIBRONECTIN FRAGMENT MEDIATED MOTILITY

6.1 INTRODUCTION

Extracellular matrix remodelling forms an essential step in both wound healing and cancer progression. Many tumours in their advanced stages show a high expression of a variety of proteases (Streuli 1999). Such proteases may not only be involved in remodelling but also in the fragmentation of molecules within the matrix forming potentially bioactive components (Streuli 1999).

There is a large body of evidence closely associating integrins and MMPs as collaborators in such processes as cancer progression and wound healing. Some integrins may modulate the action of MMPs by binding directly to them. Such interaction may enable the cell to direct proteolytic activity in the desired manner at the migrating front. After cleaving the ECM components the migrating cell can further use the integrins for attachment. Such binding of MMPs to the cells' integrins makes the latter both the activators and the targets of such pericellular proteolysis (Koistinen & Heino 2002). A positive correlation between MMPs and malignant tumours such as colon, lung, head and neck, breast and basal cell has been established (Westermarck & Kahari 1999). Recent evidence has expanded the role of MMPs from simply

degrading surrounding extracellular matrix to creating and maintaining a microenvironment that facilitates the growth and angiogenesis of tumours (Nelson *et al.* 2000). Being wide spectrum proteases, matrix metalloproteinases are able to break FN into smaller fragments (Reunanen & Kähäri 2002). Ligation between integrins and FN activate MAPK pathways via Ras/Raf-1 and p38 (Esparza *et al.* 1999) and inhibition of the MAPK pathways leads to a reduced expression of MMP-2 and MMP-9 in lymphocytes (Esparza *et al.* 1999).

6.2 OBJECTIVES

Our aim was to assess the influence of inhibitors of MMPs on the migration of cells on the 120kDa FN fragment, and the ability of MMPs to digest FN. Furthermore, to asses the effect of MAP Kinase and p38 pathways inhibitors and to co-localize the presence of these MMPs with FN and the αvβ6 integrin in the invading front of tumour islands in an *in vivo* situation.

6.3 MATERIALS AND METHODS

6.3.1 Migration Assays with TIMPs

Haptotactic cell migration was measured as previously described in chapter 2, using polycarbonate Transwell® inserts (Corning Costar). Inserts were coated the 120kDa FN fragment in PBS at a concentration of 1μM/ml and VB6 cells were seeded at a seeding density of 10^5/well. The cells were seeded in SFM containing either matrix metalloproteinase inhibitor TIMP-1 (Oncogene Research Products, Nottingham, UK) or a chemical MMPs inhibitor, Roche Inhibitor (Roche Diagnostics GmbH, Pharma Research, Lewes, UK). Cells were allowed to migrate as described in section 2.2.1.

6.3.2 Immunohistochemistry

Oral squamous cell carcinoma cases were obtained from the archives of the Oral Pathology department at UCH (Table 6.1) ensuring that each showed an area of squamous cell carcinoma invading front (Ethics Approval reference number 02/E013). Each case was stained for:

- *Cytokeratin:* Monoclonal Mouse Anti-Human Cytokeratin EA1/EA3 (Dako Cytomation, Ely, UK).
- *MMP-9:* Rabbit Anti-Human MMP-9 polyclonal antibody (Dako Cytomation).
- *MMP-2:* Rabbit Anti-Human MMP-2 polyclonal antibody (Dako Cytomation).

- *Cellular Fibronectin:* Mouse Anti-Human cellular FN (ED-A domain) (Oxford Biotechnology®, Kidlington, UK).
- *β6 integrin:* Mouse Anti-Human monoclonal αvβ6 integrin 10D5 (Chemicon International, Chandler's ford, UK).

CASES:	Location of lesion
A	Buccal Mucosa
B	Lateral border of tongue
C	Lateral border of tongue
D	Lower lip
E	Lower alveolus
F	Tongue
G	Mandible/Lip

Table 6.1 OSCC cases. Represented in this chapter are cases A and B.

Negative controls were left without primary antibodies. The procedure for MMP-2, MMP-9, cytokeratin and cFN was carried out as previously described in section 2.4.2.

The slides to be stained for beta-6 integrin were dewaxed as previously described (section 2.4.2). Endogenous peroxidase was blocked using 30% H_2O_2 in methanol (Appendix 1) and antigen retrieval was carried out using Pepsin (Zymed® Laboratories Inc., Cambridge, UK). In order to amplify the staining, biotin/avidin blocking was carried out using Vector SP-2001 (Vector Laboratories, Peterborough, UK). Avidin was applied for 10 minutes, slides

were washed twice in PBS, Biotin was applied for 10 more minutes and slides were washed again twice with PBS. To block non-specific binding 0.5%Casein/0.05%NaN_3 was added for 10 minutes (Appendix 1). The primary antibody was added to 0.1%BSA 0.1%NaN_3 in PBS on the slides and incubated overnight at 4°C.

The following day the slides were washed twice with PBS. The secondary antibody kit used was the Elite ABC reagents Vector kit PK-6102 (Vector Laboratories) and slides were washed twice in PBS between each step. The anti-mouse biotinylated secondary antibody Alexa Fluor® 488 goat anti-mouse IgG (Molecular Probes, Invitrogen Detection Technologies, Paisley, UK) was applied for 30 minutes at room temperature. Slides were stained using the DAB substrate kit Vector SK-4100 (Vector Laboratories) for 5 minutes. Slides were rinsed under running water for 2 minutes, stained with Mayer's haematoxylin for 1 minute, rehydrated and mounted as described in section 2.4.2.

6.2.3 Digestion of pFN

Pro-MMP-2 and pro-MMP-9 were obtained from Calbiochem® (VWR International Ltd., Poole, UK). Prior to running the digestion experiments the pro-MMPs were activated using organomercurial stock solution. P-amino phenylmercuric acetate solution was added to 0.1M NaOH solution just prior to incubation with the pro-MMPs. Pro-MMPs were added to this stock

solution at a ratio of 10:1 and incubated at 37°C for 2-3 hours. Following activation, MMP-2 and MMP-9 were incubated with pFN (10µg/ml) at a ratio of 1:25 (MMP-2) and 1:10 (MMP-9) (varying concentrations of both MMP-2 and MMP-9 to pFN were tested to decide the optimal ratio to use in our work, (Appendix 2). The pFN/MMP mixtures were incubated at 37°C for 2 hours, 6 hours, 24 hours and 48 hours, after which 20µl was removed from the mixture and added to non-reducing buffer X2 (Appendix 1) to immediately stop the reaction.

The samples were loaded on an SDS 10% Acrylamide Gel and run as previously described in section 2.3.2. The gels were then stained with commassie blue and an image taken with Alpha imager 1220 version 5.5 (Alpha Innotech Corp™).

6.2.4 Intracellular signalling pathways

Experiments were performed to block two intracellular signalling pathways implicated in tumourigenesis, in order to investigate their role in modulating MMP production by VB6 cells. U0126 inhibits MEK1 and MEK2 of the MAP kinase pathway, and SB 203580 inhibits the SAPK2 (p38) cascade (Sigma-Aldrich Company Ltd.). Dose response curves were carried out to determine optimal concentrations without detrimentally damaging the cells as previously reported by Dalvi *et al.* (unpublished data).

Cells were seeded in 24-well plates (Nunc™, VWR International) previously coated with pFN and 120kDa FN fragment in Defined Keratinocyte SFM (Invitrogen Corporation, Gibco™) for 4 hours. The cells were then washed twice with PBS, and medium containing 100µM U0126 and 20µM SB 203580 was added to the different samples. Cells were incubated overnight at 37°C. The following day zymograms were run of the supernatants as previously described (section 2.3.3) and the data collected was analysed with Scion Image (Scion Corporation).

6.4 RESULTS

6.4.1 Migration on the 120kDa FN fragment is not reduced by adding MMP blockers

We have previously described in chapter 4 the upregulated migration of beta-6 expressing cells on the 120kDa FN fragment. We have also shown evidence of increased MMP expression by cells seeded on the fragment in chapter 5. Having established the importance of the FN-binding integrins in the upregulated migration towards the 120kDa FN fragment, we attempted to inhibit such migration using matrix metalloproteinase inhibitor TIMP-1 (Oncogene Research Products) and a chemical MMPs inhibitor, Roche Inhibitor (Roche Diagnostics GmbH) (Figure 6.1 and 6.2).

Both TIMP-1 and the Roche inhibitor showed a trend towards lower migration of VB6 and C1 migration on the 120kDa FN, but this was not statistically significant (p value= 0.1 for C1 and 0.7 for VB6+TIMP-1, Mann-Whitney test, and p value= 0.2 for C1 and 0.1 for VB6+RO, Mann-Whitney test).

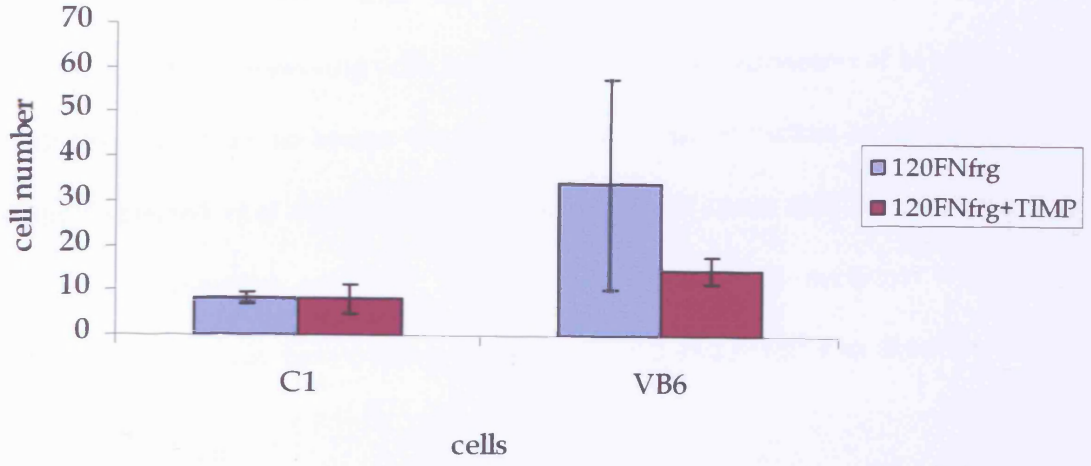


Figure 6.1 Migration assay of C1 and VB6 on 120kDa FN fragment and on the fragment plus TIMP-1.

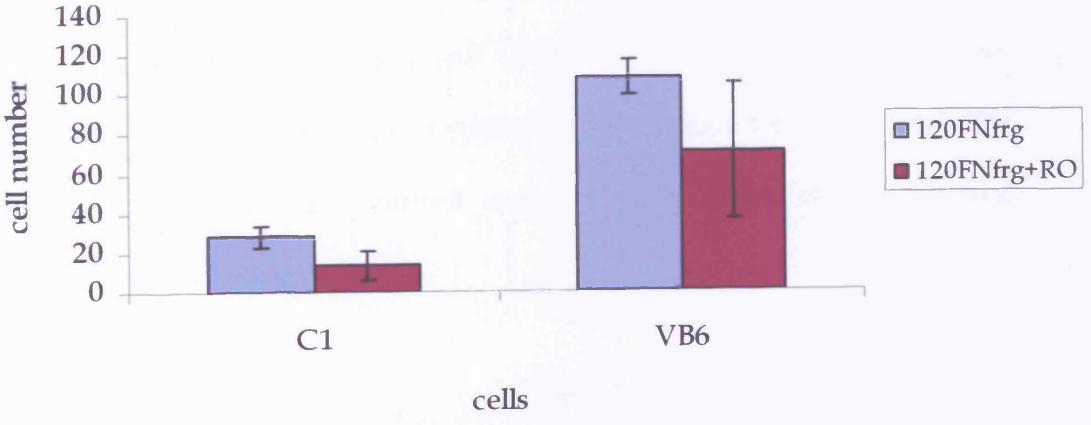


Figure 6.2 Migration assay of C1 and VB6 on 120kDa FN fragment and with MMP Roche Inhibitor.

6.4.2 MMP-2 and MMP-9 co-localize with beta-6 integrin and cellular FN around the invasive front of SCC lesions

In our work, we showed that the cell-binding FN fragment enhances migration of beta-6 expressing cells and upregulates the expression of MMP-2 and MMP-9. In order to assess the localization of these factors *in vivo*, we randomly selected oral squamous carcinoma tumour cases and carried out immunohistochemistry analysis. The paraffin embedded sections were stained for cytokeratin, beta-6 integrin, cFN, MMP-2 and MMP-9 as described in section 2.4.1.

Positive cytokeratin staining was obtained in all sections and was specific to keratinocytes (Figure 6.3 and 6.5 (A)). Cellular FN staining was observed in the stroma surrounding the epithelial tumour island in a fibrous form (Figure 6.3 and 6.5 (B)). MMP-2 and MMP-9 were observed both within the tumour island as well as in the surrounding stroma, MMP-2 staining being more intense (Figure 6.3 and 6.5 (C) and (D)). Beta-6 integrin was seen to be expressed on the cell membrane of epithelial cells (Figure 6.4 and 6.6 (A)). The negative control with the omitted antibody was devoid of any staining (Figure 6.4 and 6.6 (B)).

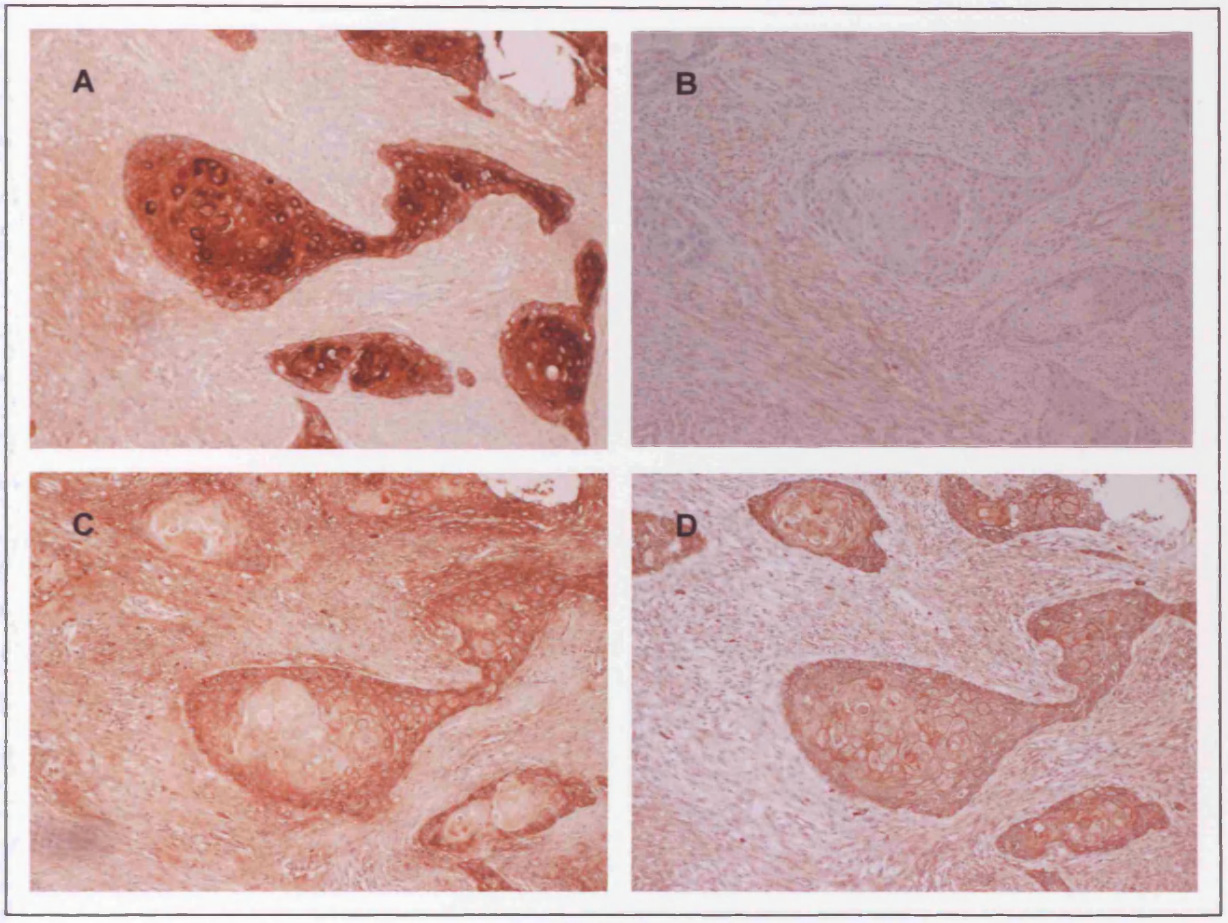


Figure 6.3 Epithelial tumour island showing intense staining for cytokeratin (A), light staining fc cellular fibronectin, in the tumour stroma (B), intense MMP-2 staining within the tumour cells and the surrounding stroma indicative of a progressive and highly invasive tumour (C), and MMP-9 staining, not as intense as MMP-2 but can also be seen in and around the tumour island (D) (Magnification x200).

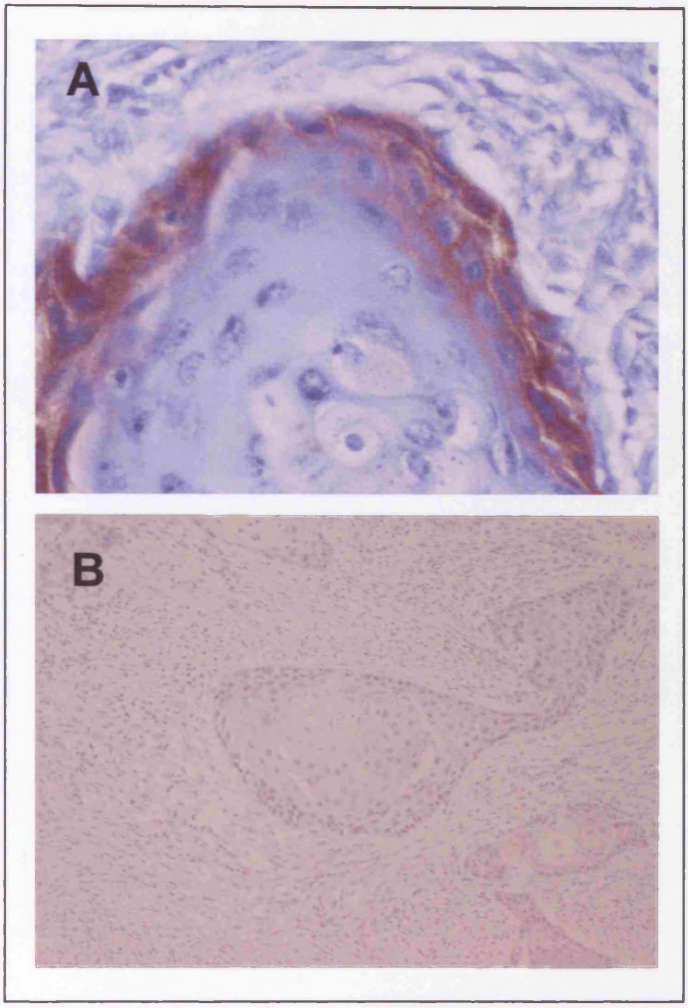


Figure 6.4 Section (A) viewed under high power shows beta-6 integrin staining along the invading front of the epithelial tumour cells (Magnification x 400 ; section (B) is of the negative control showing haematoxylin staining devoid of any other staining (Magnification x 200)

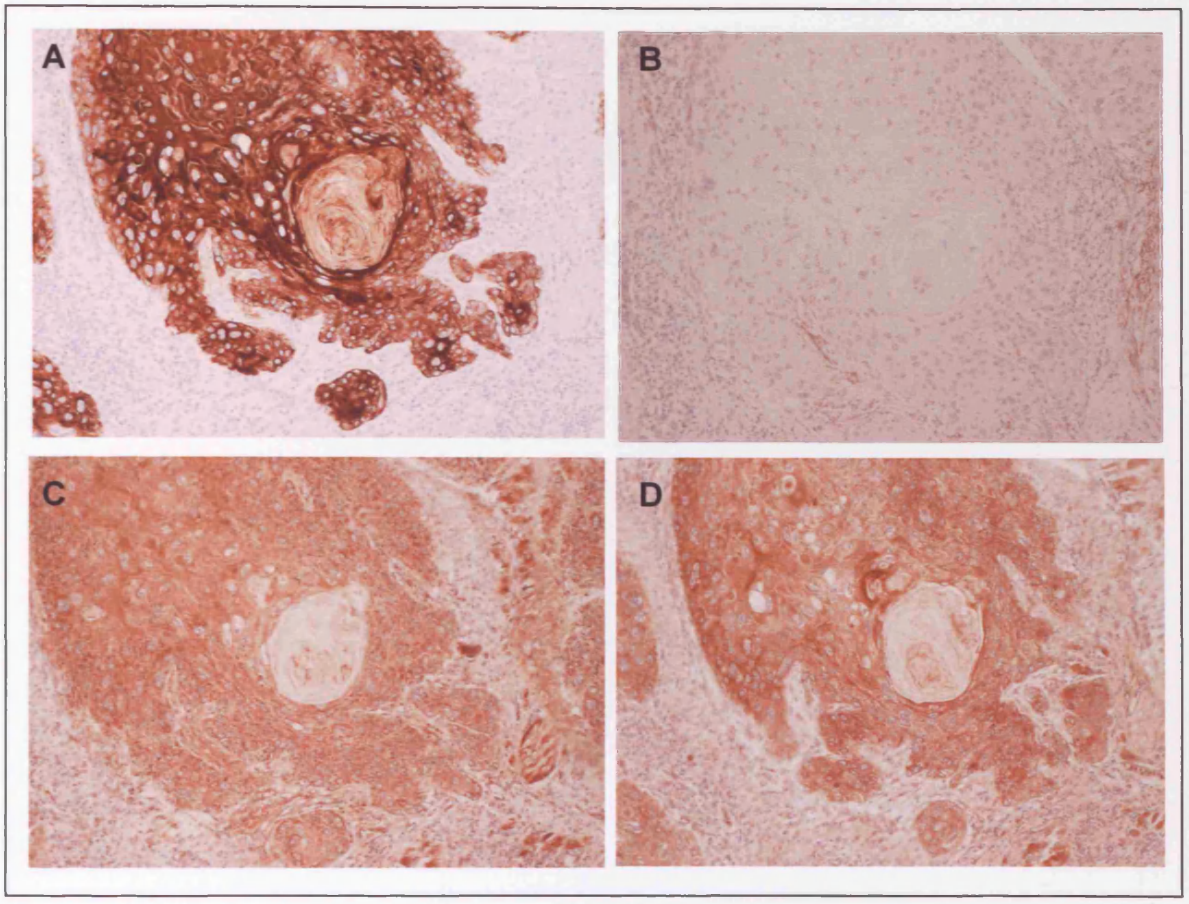


Figure 6.5 Another case of epithelial tumour island showing intense staining for cytokeratin (A), light staining for cellular Fibronectin, in the tumour stroma (B), intense MMP-2 staining within the tumour cells and the surrounding stroma indicative of a progressive and highly invasive tumour (C), and MMP-9 staining, not as intense as MMP-2 but can also be seen in and around the tumour island (D) (Magnificat x200).

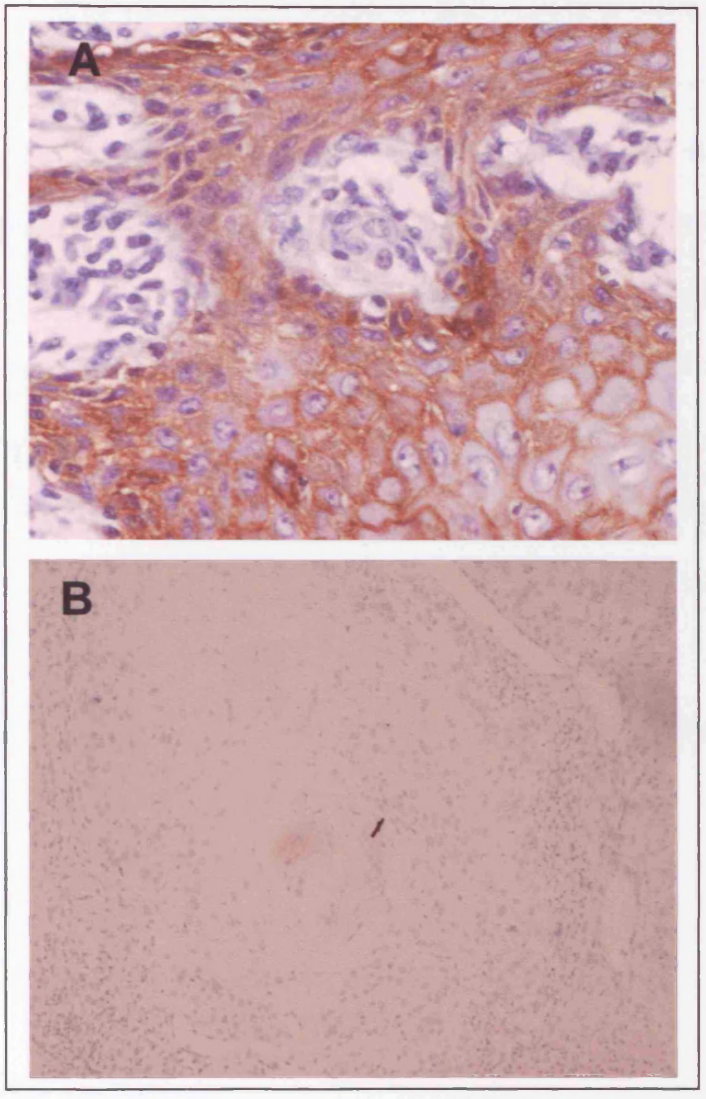


Figure 6.6 Section (A) viewed under high power shows beta-6 integrin staining along the invading front of the epithelial tumour cells (Magnification x 400); section (B) is of the negative control showing haematoxylin staining devoid of any other staining (Magnification x200).

6.4.3 MMPs are able to degrade pFN into various size fragments in a time-dependent manner

Active MMP-2 and MMP-9 were incubated with plasma FN and analysed over time to assess proteolytic degradation. Fibronectin is a known substrate of many proteases and in this particular case we tried to assess the various molecular weight fragments generated. At and after 24 hrs there is a clear band forming at the level of the 120kDa FN fragment, suggesting that at this time point the fragments generated by MMP-9 include the cell-binding FN fragment. The initial attack on pFN may simply divide the diameric protein into its two principal 200-220kDa fragments. Following this initial breakdown the two fragments may then be digested more thoroughly to yield smaller fragments. In our result it is noticeable that MMP-9 degrades FN to a higher degree than MMP-2 (Figure 6.7 and 6.8) and the generation of fragments in this case is more evident.

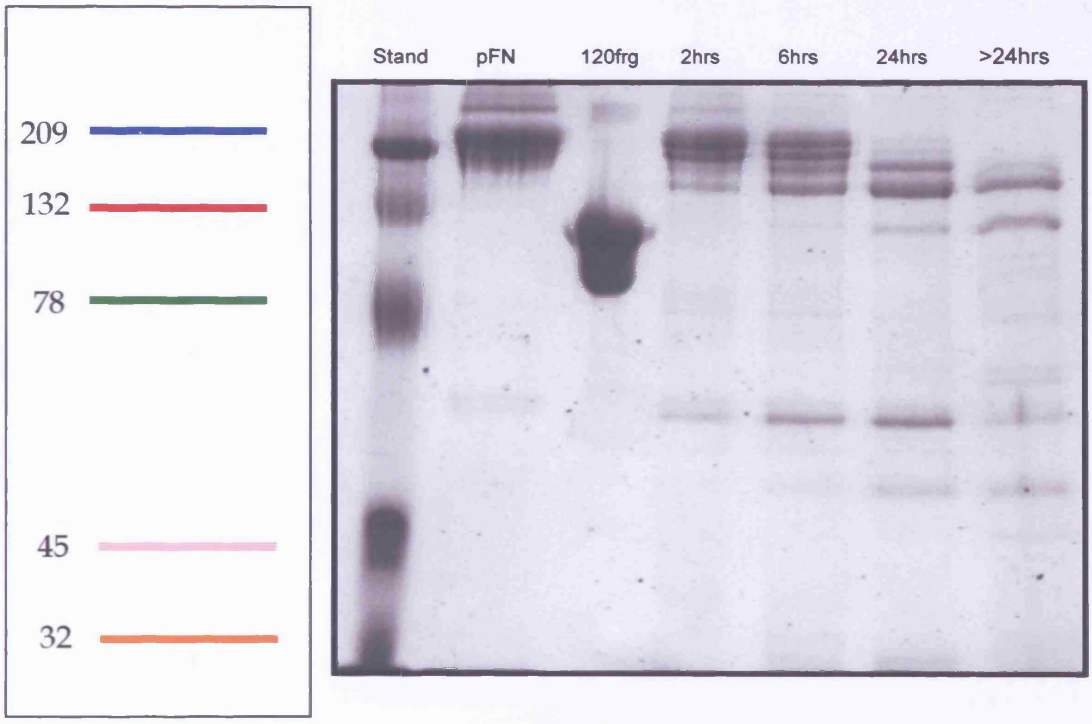


Figure 6.7 Digestion of pFN by MMP-9 over a period of over 24 hours. The gel clearly shows progressive degradation of pFN over time producing more and more fragments. At 24hrs and above the cell-binding fragment is clearly visible.

6.4.4 Blocking MEK1 and MEK2 of the MAP kinase and SAPK2 (p38) reduces the expression of MMPs by cells plated on the fragment

Inhibitors to the MEK1 and MEK2 of the MAP kinases pathways, and SAPK2 (p38) were added to cells plated in SFM. MAP kinase and p38 are triggered by the binding of integrins to FN (Esparza *et al.* 1999) and this leads to signalling which triggers the expression of MMPs (Figure 6.9).

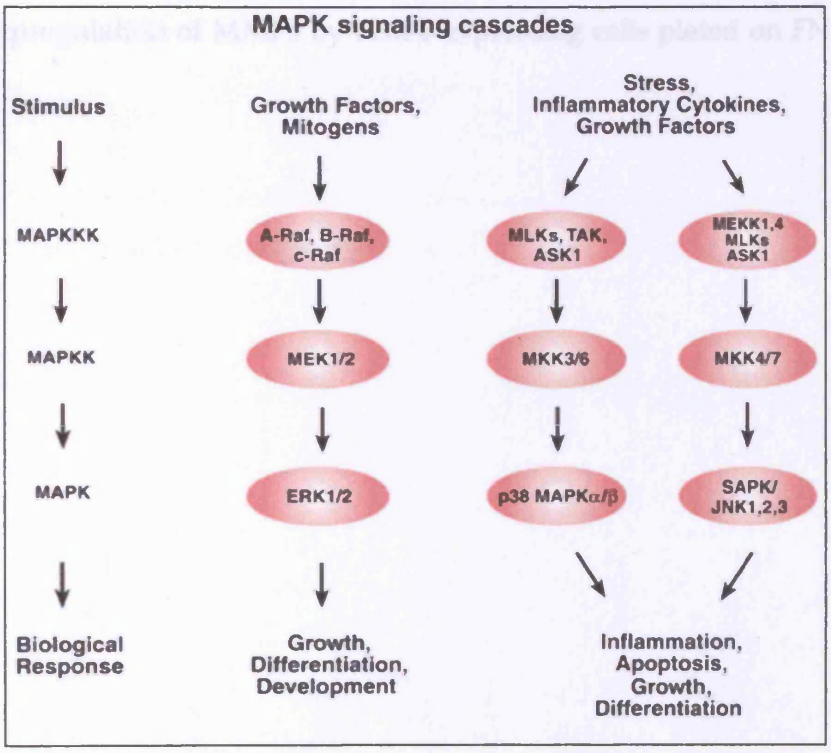


Figure 6.9 Diagrammatic representation of various MAPK intracellular signalling pathways (Sigma Cell Signalling catalogue).

The inhibitors were added to VB6 cells plated on pFN and the 120kDa FN fragment for 24 hours and the conditioned medium was then collected and subjected to zymography. Our results have shown a downregulation of

MMP-2 and MMP-9 produced by VB6s exposed to these inhibitors as opposed to the control. The down regulation of MMP-9 is apparent when blocking MEK whereas MMP-2 does not seem to be affected (Figure 6.10). However, blocking p38 seems to have an effect on both MMP-9 and MMP-2 on both the intact pFN and the fragment (Figure 6.11).

This is indicative of the importance played by these two signalling pathways in the upregulation of MMPs by beta-6 expressing cells plated on FN and its fragment.

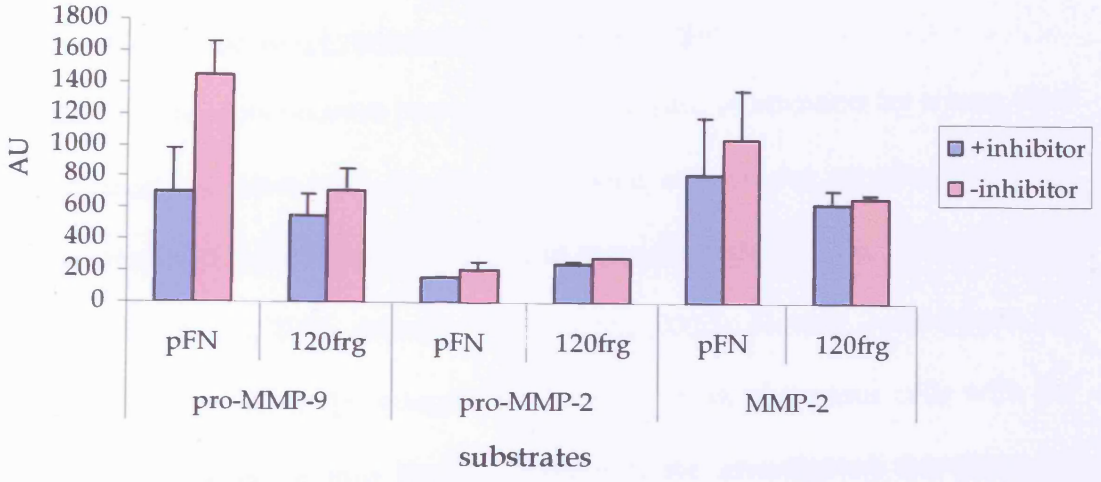


Figure 6.10 Bar chart clearly shows a down-regulation of pro-MMP-9 in the Presence of SB203580, the p38 inhibitor.

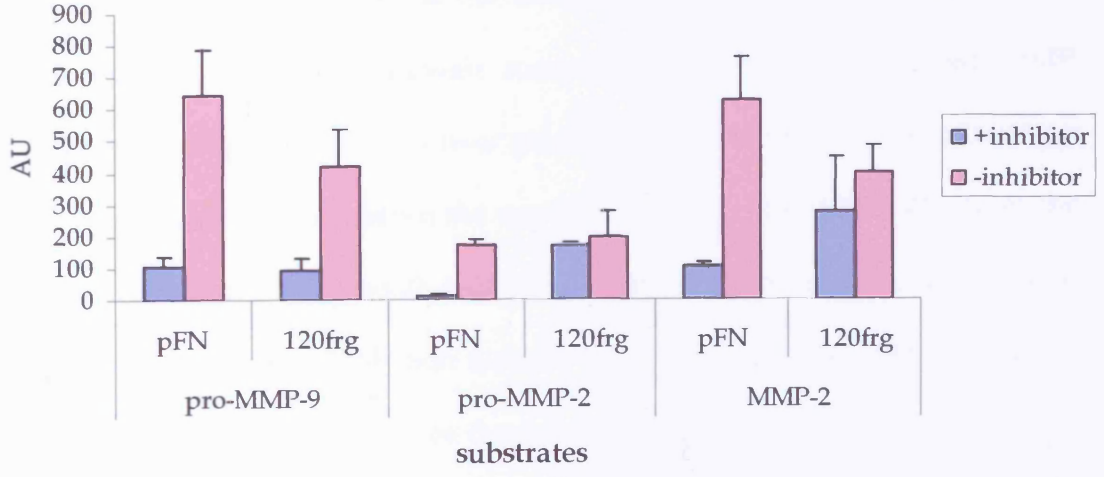


Figure 6.11 Bar chart showing a down-regulation of pro-MMP-9 and MMP-2 in thepresence of U-0126, a MEK1 inhibitor.

6.5 DISCUSSION

6.5.1 Matrix metalloproteinases and their inhibitors

Matrix metalloproteinases have been at the centre of attention for a long time and many reviews and studies have been carried out to elucidate their importance in both wound healing and tumourigenesis (Chang & Werb 2001; Liotta & Kohn 2001; Mueller & Fusenig 2002). Having established the importance of the αvβ6 integrin in the interaction of tumour cells with the intact FN molecule and the FN fragment, we investigated the effects of various MMP inhibitors in the upregulated migration of VB6 cells towards the 120kDa FN fragment.

TIMPs expression has been observed in the tumour stroma and their over-expression can be viewed as the cells' attempt to regulate the MMP over expression. However, although many have found that increased TIMP expression is indicative of a lower grade tumour (Polette *et al.* 1991; Tsuchiya *et al.* 1993), some have shown the opposite (Visscher *et al.* 1994). Whatever the prognosis, there is general consensus that TIMP expression increases in tumours as opposed to non-tumour sites (Yoshiji *et al.* 1996). But, the determining factor seems to be the ratio of MMP:TIMP expressed by tumours (Yoshiji *et al.* 1996). TIMPs appear to have ambiguous effects on tumour cells and this could be explained by the fact that as well as inhibiting MMPs they serve as activators of MMP pathways (Gomez *et al.* 1997). Our studies failed

to show a down-regulation of migration in the presence of MMP inhibitors and this could reflect the fact that the MMPs produced by tumour cells may be evading otherwise common regulatory signals. Alternatively, lacking other important cues TIMPs may be simply acting by activating MMPs. However, it could also be that MMPs in their zymogen form are not inhibited by TIMPS.

6.5.2 MMP-2, MMP-9, the αvβ6 integrin and fibronectin in the invading front of tumours

The interaction between integrins and the MMPs may be one of the key phenomena in the invasion process (Koistinen & Heino 2002). The importance of the αvβ6 integrin in the expression of MMP-9 by tumour cells has been previously described and was found to co-localize with the invading front of tumours concurrently where the MMPs are concentrated (Thomas *et al.* 2001b). Thomas *et al.* (2001b) showed increased expression of MMP-2 and MMP-9 by cells expressing the αvβ6 integrins when plated on FN. This group also showed that adding blocking antibodies to the αvβ6 integrin greatly reduced expression of MMP-9 in normal human keratinocytes. Gu *et al.* (2002) have shown that colon cancer cells transfected with the β6 integrin expressed higher amounts of MMP-9 as compared to the same cells transfected with the null transfectant. This was further investigated by Morgan *et al.* (2004) who identified the cytoplasmic-tail motif EKQKVDLSTDC of the αvβ6 integrin as being responsible for this integrin's invasive potential through the upregulation of MMPs. Monsky *et al.* (1993) provided evidence that suggests

MMP-2 production by stromal cells that then become attached to the invading front. Vaisanen *et al.* (1998) also reports an increased expression of MMP-2 with increasing tumour grade whereas in melanomas MacDougall *et al.* (1995) show that the expression of MMP-9 is associated with metastasis.

We were able to show the presence of the αvβ6 integrin in tumour sections and by locating MMP-2, MMP-9 and cFN in the stroma of the tumour island we can suggest the hypothesis that these molecules are interacting with each other *in vivo*. The MMPs produced in and around tumour lesions may be proteolytically degrading the extracellular matrix generating proteolytic fragments. This firstly promotes degradation of the extracellular matrix that facilitates the tumour cells physical invasion and metastasis. Secondly, the resultant proteolytic fragments may in turn give further "malignant" signals and stimulate cell migration and invasion. Such stimulation is in many cases confined to the invading front at the forward edge of invading cells, where an array of enzymes, inhibitors and receptor molecules go through cycles of activation, deactivation and so on (Liotta & Kohn 2001).

6.5.3 Tumour stroma and MMPs

In our immunohistochemistry analysis of tumour lesions we have described the distribution of MMPs both in the tumour islands and in their surrounding stroma. This is in agreement with previous findings that indicate that MMPs and proteases involved in tumour progression may be secreted by stroma

cells rather than the tumour cells themselves. Thomas *et al.* (2002) have shown that tumour cells are able to utilize MMPs produced by stromal cells. Shapiro *et al.* (1998) has shown that MMP-2 is often produced by tumour cells but other MMPs and proteases involved in invasion are more likely secreted by stromal and inflammatory cells. Mueller *et al.* (2002) have shown that the increased expression of MMP-9 comes from stromal cells surrounding the tumour and this may in turn upregulate the expression of MMPs in the tumour cells too. MMP-2 has been correlated with malignant transformation of epithelial cells (Davidson *et al.* 1999; Talvensaari-Mattila *et al.* 1999) and is expressed by many normal as well as transformed cells, whereas MMP-9 is expressed by keratinocytes, monocytes, alveolar macrophages, PMN leukocytes and many malignant cells. Basset *et al.* (1997) have put forward a simple model of malignant cells-stromal cells interactions by describing MMP-2. This protease has been found to be secreted copiously by tumour cells; however, its activator, MT-MMP is expressed solely by stromal cells. Therefore, MT-MMP attached to stromal cells attracts the secreted MMP-2 and activate it forming a synergistic interaction with the invading tumour cells. Similarly the uPA system follows a comparable pattern where the urokinase is secreted by stromal cells and its receptor uPAR is secreted by tumour cells (Basset *et al.* 1997).

MMPs are activated in the extracellular space and their expression in tumours is tightly regulated. It has been suggested that there is a continuous cross-talk

between tumour cells, stromal cells and inflammatory cells during active invasion. It has been shown that the MMPs involved in a particular tumour are produced by stromal cells such as fibroblasts and inflammatory cells rather than tumour cells (Nelson *et al.* 2000). Therefore, stromal cells may participate in the tumourigenic conversion of epithelial cells. Such evidence is further confirmed by the localization of MMPs at the invading front both in tumour cells and stromal cells. It is in fact this cross-talk that may play a potentially vital role in the activation of MMPs (Westermarck & Kahari 1999).

Evidence now points to the importance of the tumour cell-stroma interaction in the regulation and maintenance of the invasion and progression of the tumours (Mueller & Fusenig 2002). Previous studies have shown the presence of MMPs in the stroma immediately surrounding tumour islands, in particular around the invading front (Kikuchi *et al.* 2000; Gu *et al.* 2002). Tumour cells may induce a specific "malignant" type of stroma around the lesion which has lost controlling functions and gives cues to enhance the proliferation and invasion capabilities of malignant cells (Mueller & Fusenig 2002). The stroma surrounding the tumour cells may in effect be the conductor in an "orchestra" of invasion and progression. Local attractants derived from the stroma include degraded matrix proteins.

6.5.4 Role of uPA

In chapter 5 our results showed an upregulation in MMP production but failed to show the same for uPA. However, it would be premature to exclude this serine protease from the fragment-MMP cascade. It may in fact be that the serine protease, though not upregulated in presence of the fragment, acts to activate the MMP and thus be playing a secondary, albeit important, role. This was shown by studies that found the activation of pro-MMP-9 to active MMP-9 being regulated by plasmin and stromelysin, both found abundantly in tumours. It was found that PAI-2, a uPA inhibitor when added to colon cancer cells reduced collagenases degradation by such β6-expressing cells; this can be explained by the need for uPA to convert plasminogen into plasmin which then converts pro-MMP-9 into active MMP-9 (Thomas *et al.* 2002). Such data not only confirms that tumour-associated proteolysis is a cell surface event and may be important in the generation of an invasive phenotype (Agrez *et al.* 1999), but further confirms the synergy between different proteases. While tumour cells may be only expressing specific proteases, in our case, MMPs, the surrounding stroma cells may in fact be secreting the proteases that enable the former to act. This is reaffirmed by the fact that plasmin mediates MMP activation (Shapiro 1998), and that Thomas *et al.* (2001a) have shown inhibition of MMPs when adding a uPA inhibitor.

The urokinase plasminogen activator role in the activation of MMPs doesn't have to be a direct one but could be working in a more indirect fashion

through the activation of plasminogen. The conversion of plasminogen to plasmin may in itself activate MMPs that in turn activate the main MMPs involved in the extracellular matrix degradation. This example has been illustrated by Ramos-DeSimone *et al.* (1999) who showed a link whereby pro-MMP3 was activated by plasmin and active MMP-3 then activated pro-MMP-9. In this case uPA was necessary to convert plasminogen into plasmin. Furthermore, they speculated that pro-MMP-3 was likely to be secreted by stromal cells surrounding the tumour rather than the tumour cells themselves.

Although we have suggested a role played by uPA in the activation of MMPs, other proteases may also be playing a part. Esparza *et al.* (1999) showed evidence that MT-MMP is an important activator of MMPs, and in human lymphocytes they showed that FN was able to induce MMP-9 and MMP-2 as well as promoting MMP-2 activation by upregulating MT-MMPs. Immunohistochemical studies have found that increased expression of MT-MMP and MMP-2 correlates with invasion-positive cases in colorectal carcinoma. This supports, along with the localization of MMP-2 around cancer cells, the suggestion that MT-MMP is essential in the activation of MMP-2 (Kikuchi *et al.* 2000). The activation of pro-MMP-2 may be brought about by MT-1MMP whereas the activation of pro-MMP-9 requires MMP-3.

6.5.5 Fibronectin as a substrate for MMP-2 and MMP-9

Having co-localized FN, the αvβ6 integrin, MMP-2 and MMP-9 to the invading front of tumours, and having postulated the proteolytic action of these MMPs on the intact FN, we assessed this by carrying out digestion experiments. Evidence of the proteolytic action of MMPs on other extracellular matrix molecules has already been investigated. Giannelli *et al.* (1997) reported that laminin-5 cleavage by MMP-2 caused increased migration of epithelial breast cells. It seems that cleavage of this substrate by MMP-2 exposes pro-migratory cryptic sites within the molecule. Fukai *et al.* (1995) have shown that digestion of FN by MMPs in particular MMP-2 releases various fragments that possess strong chemotactic properties. Our digestion experiments have shown that MMP-9 in particular can degrade FN to produce fragments including the pro-migratory 120kDa fragment in a time dependent manner. MMP-9 can cleave FN and gelatin to expose the RGD site which is then recognized by the αvβ6 integrin and this seems to be pivotal for the progression of the tumour (Agrez *et al.* 1994).

6.5.6 MMPs and intracellular signalling pathways

Finally, attempts were made to extend the studies described above to include preliminary signalling pathways data. Ligation between integrin and FN activate MAPK pathways via Ras/Raf-1 and p38. Esparza *et al.* (1999) have shown that inhibition of the MAPK pathways leads to a reduced expression of MMP-2 and MMP-9 in lymphocytes. Their findings suggest a signalling

pathway within the cell by which MMPs regulate tight ECM degradation around the cells upon contacting FN. Our study has shown a down-regulation of both MMPs when blocking the p38 pathway, showing that it is common to both proteases. However, blocking the MAPK pathway at the level of MEK1/2 downregulated MMP-9 but not MMP-2. It seems that this pathway is not shared and MMP-2 signalling follows another route. This was shown by Wang *et al.* who treated neuronal cultures with both SB203580 and U0126 to assess their effects on MMP-2 and -9 production and found that when both inhibitors worked together there was significant reduction in MMP-9 production. Using either inhibitor alone there was little effect. This shows the presence of possible redundancy in either pathway in respect with MMP-9 production (Wang *et al.* 2002). Cho *et al.* (2000) showed the same results when using the same inhibitors on MMP-9 expression by vascular smooth muscle cells.

6.5.7 Conclusion

Previous studies along with our findings have brought together enough evidence to suggest that integrins, MMPs and FN fragment act in synchrony to maintain a milieu that enables malignant cells to survive and enables wounds to heal. It is this intricate highway of cues and signals that turns on and off the switches needed to effectively allow degradation of the extracellular matrix, detachment of cells and migration. In tumourigenesis

this is coupled with the impediment of normal regulatory mechanisms from restraining and placing order to a somewhat chaotic scenario.

CHAPTER 7

DISCUSSION

During both wound healing and tumourigenesis epithelial cells acquire a more mesenchymal phenotype. This enables them to move freely and change in function as the situation necessitates. They can breach barriers, secrete proteases when needed and escape regulatory mechanisms such as apoptosis. During tumourigenesis, cancer cells exist under a new law and act independently of protective mechanisms. Epithelial cells interact with surrounding stroma cells and induce them into promoting a suitable environment that induces further invasion. It is such interactions with the extracellular matrix that ensures the immortality of the tumour. Changes in stromal behaviour can influence epithelial transformation and phenotype. Activation of local stroma is parallel with the shift from normal to invasive carcinoma and an environment that stimulates cancer invasion is created. The enzyme cascade of malignant cells is confined to the leading edge of the tumour cell (Liotta & Kohn 2001).

Tumour cells secrete proteases that breakdown the surrounding extracellular matrix. It is now well proven that this isn't just a structural breakdown that creates "pathways" for tumour cell to migrate through, but rather, it is a dynamic process whereby bioactive molecules are released. Extracellular

matrix molecules can gain new biological functions when partially degraded due to their modular make up (Hynes 1992) and therefore possess functions that are absent in the parent molecules. Extracellular matrix molecules such as collagens and laminins and their respective fragments have already been extensively investigated as mentioned in section 1.3, and although FN fragments have not received similar attention, they are found in abundance in the ECM and their correlation with tumourigenesis has been suggested (Schedin *et al.* 2000). An example of such a fragment is migration stimulating factor (MSF), a 70kDa FN fragment containing of the peptide sequence of the gelatin-binding domain that has been shown to be produced only in foetal, cancer and activated keratinocytes. This fragment is not generated in normal adult human cells and its effects range from inducing migration, angiogenesis and hyaluronan acid synthesis (Schor *et al.* 2003). In our work we chose to investigate the 120kDa cell-binding FN fragment. The relationship between β6 expressing cells and FN fragments was of particular interest to us. Although tumours express a number of FN binding integrins including α5β1, the data presented in this thesis indicates that the 120kDa cell-binding FN fragment only exhibits its effects on cells expressing β6 via the RGD sequence. We believe such interaction is responsible for the increased migration we have observed over the fragment. Therefore, since this integrin is expressed *de novo* in cancer (and wound healing), the effect is specific to the integrin.

In our study we have found αvβ6 expressing cells to be more migratory over the 120kDa cell-binding FN fragment than the intact plasma FN. The proposed mechanism behind this increase in migration is an increased production of proteases. While increased production of the urokinase type plasminogen activator was not observed, increased production of MMPs -2 and -9 was. Both MMPs are known to degrade extracellular matrix molecules. Furthermore, FN is a known substrate of matrix metalloproteinases and it seems likely that a loop exists that is initiated when the tumour violates regulatory mechanisms and assumes an invasive state. We believe that such tumour cells under the effect of extracellular matrix by-products (which include FN fragments) produce more MMPs which breakdown surrounding extracellular matrix and generate FN fragments which, via the β6 integrin, not only degrade the surroundings, but also continue the degradation of FN and the generation of more bioactive fragments (Figure 7.1). Our immunohistochemistry experiments showed the co-localization of FN, the αvβ6 integrin, MMP-2 and MMP-9, and give further credence to the suggested loop occurring in *in vivo* situations.

In our work the urokinase-type plasminogen does not appear to be upregulated when the FN cell-binding fragment interacts with β6 expressing cells. However, this does not exclude its well established importance in tumourigenesis (Andreasen *et al.* 1997; Reuning *et al.*1998; Stoppelli 2005). In fact, the MMPs secreted are in an inactive state and are activated by plasmin.

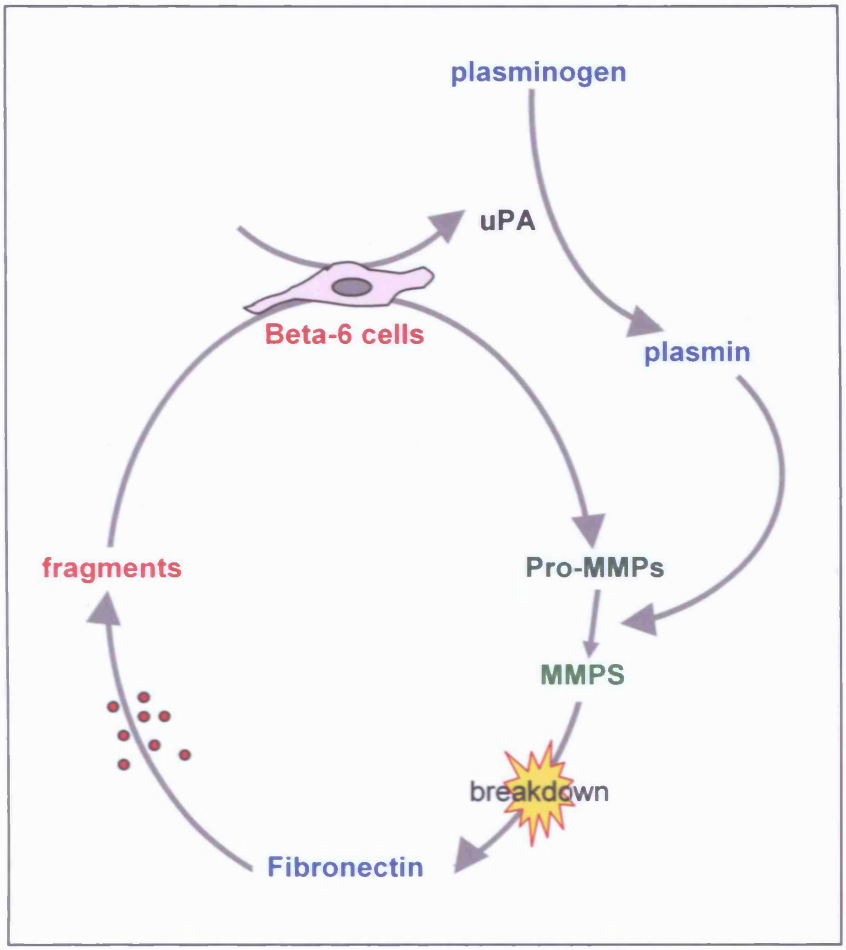


Figure 7.1 Diagrammatic representation of the positive feedback loop; β6 expressing cells when exposed to breakdown products of the ECM secrete pro-MMPs and uPA. The latter converts plasminogen into plasmin which in turn activates pro-MMPs. Activated matrix metalloproteinases then further breakdown ECM molecules (such as fibronectin) to generate more bioactive bi-products.

Plasmin is formed from plasminogen under the effect of uPA. Beta-6 transfected cells have been found to produce more uPA which may be vital for the conversion of MMPs into active enzymes. However, although this upregulation is not affected by the fragment or whole molecule, the uPA produced by such cells may play an active role in their migration by activating MMPs.

A lot of attention has been recently given to the relationship between the various cell adhesion molecules. In our work, when establishing the optimal conditions in which to carry out the various functional assays we noticed an alteration in the cell morphology when such cells were starved of certain nutrients. When investigated further there was a suggestion of a link between the expression of E-cadherin and the presence of EGF. It is easy to assume an inverse relationship between the presence of E-cadherin and the β6 integrin. While the former is common in the less aggressive lesions, the latter is a marker of aggressive tumours. Could there be cues in the tissue microenvironment that promote one over the other? In the absence of EGF cells seemed to revert to a more epithelioid appearance and there is a suggestion of E-cadherin upregulation. Cell-to-cell contact seemed to mimic normal epithelia and there were less signs of migratory armamentaria such as lamellopodia and filipodia. Interestingly, work by Chunthapong *et al.* (2004) showed that E-cadherin is broken down to smaller fragments by the action of MMPs in prostate cancer cell lines. Such fragments have been found to

promote a more aggressive phenotype as opposed to the intact E-cadherin. If added to the model we have proposed for our work, the MMPs produced by our β6 cells can not only breakdown the surrounding ECM molecules, but also breakdown the E-cadherin present on the cells to: 1) decrease the expression of E-cadherin and 2) to form bioactive by-products that further reinforce the aggressive nature of the cells. It is clear that complex processes such as cell migration and differentiation need an integrated response to multiple external stimuli an example being the cross regulation among cell adhesion molecules. This could create a paradigm shift; instead of manipulating cells to revert back to their original state we could manipulate the environment to swing the balance and encourage the reversal to more benign phenotypes.

Wound healing and tumourigenesis are complex mechanisms; while the former is under strict regulatory mechanisms that ensure its commencement and conclusion, the latter is, in many aspects, wound healing gone awry. It is such intricate mechanism that renders cancer therapy so difficult to achieve. Although cancer is multifaceted, the role of FN fragments may be central and, being indicative of a number of tumourigenic processes, may be a useful marker of aggressive disease or therapeutic target.

FUTURE WORK

The work in this thesis has shown the cell-binding FN fragment to promote migration and MMP production in β6 expressing cells. Further studies will include localizing the exposed epitope present in the 120kDa cell-binding FN fragment (but not in the intact molecule). This will enable us to locate the fragment during immunohistochemistry, study in more detail its association with other cancer-promoting molecules and assess its localization in the lesion (for example, at the invading front). It may also be possible to study the prevalence of this fragment with various carcinoma types, such as verrucous or ulcerative lesions, early lesions or advanced ones.

Further mapping experiments to determine the active part of the 120kDa FN fragment, and the signaling pathways initiated by it will be carried out. Inhibitors of various signaling pathways may be used to determine ways to inhibit the actions evoked by this fragment.

Using the work presented in this thesis as a model, more experiments may be carried out to determine whether integrin specific effects of matrix fragments occur in other systems such as α9 integrin and Tenascin C.

Future work will also aim at assessing the relationship between EGF, cell morphology and cell behaviour. More experiments such as indirect immunofluorescence in the presence and absence of EGF may be carried out looking in particular at the distribution of E-cadherin and the appearance of the actin cytoskeleton. Migration assays may be used to assess the motility of cells grown in medium containing EGF and medium devoid of it. Time-lapse movies can provide further assessment of both morphology and motility of cells in the presence or absence of EGF. E-cadherin positive cell lines may be used as comparison to assess if β6 cells grown in the absence of EGF behave similarly.

APPENDIX 1

MEDIA, SOLUTIONS AND BUFFERS

A 1 Culture media and solutions

A 1.1 Keratinocyte growth medium

- 3 parts Dulbecco's modified Eagle's medium (DMEM) with sodium pyruvate and 1000mg/L glucose (Gibco Invitrogen, Paisley, UK)
- 1 part nutrient mixture F-12 (HAM) with L-glutamine (Gibco Invitrogen)
- 10% Foetal calf serum (PAA Laboratories, Yeovil, UK)
- 10ng/ml epidermal growth factor (Sigma-Aldrich Company Ltd., Dorset, UK)
- 0.5µg/ml hydrocortisone (Sigma-Aldrich Ltd.)
- 5µg/ml Insulin (Sigma-Aldrich Ltd.)
- 1.8×10^{14}M Adenine (Sigma-Aldrich Ltd.)
- 10^{-10}M Cholera Toxin (Sigma-Aldrich Ltd.)
- 100IU/ml Penicillin (Gibco Invitrogen)
- 100µg/ml Streptomycin (Gibco Invitrogen)
- 2.5µg/ml Fungizone (Amphotericin B) (Gibco Invitrogen)

A 1.2 Washing medium

PBS without calcium and magnesium (Gibco Invitrogen)

A 1.3 Migration

Dulbecco's modified Eagle's medium (DMEM, Gibco Invitrogen) with sodium pyruvate and 1000mg/l glucose

0.5% heat-inactivated BSA

A 1.4 Adhesion Assay

1mM $CaCl_2$

0.23mM $MgCl_2$

in PBS at pH 7.4

A 2 Immunohistochemistry

A 2.1 Harris' haematoxylin

1mg of haematoxylin

10ml of alcohol

20g of aluminium potassium sulphate

0.5g of mercuric oxide

8ml glacial acetic acid

A 2.2 Citrate buffer

0.02M Sodium Citrate

3.4mM HCl

A 2.3 Tris Buffer Saline (TBS)

1.4M NaCl

0.05M Tris

4.4mM HCl

adjust pH to 7.4 with 1M HCl

A 3 Immunofluorescence

A 3.1 Paraformaldehyde 4%

4g of paraformaldehyde

50ml dH_2O

A pellet of NaOH

The solution was placed in a water-bath at 60°C until dissolved, then 50ml 2X PBS was added.

A 3.2 Blocking solution (0.1/0.1 solution)

0.1% NaN_3

0.1% BSA

in PBS

A 4 Electrophoresis gels, solutions and buffers

A 4.1 Lysis buffer

1% SDS

10mM Tris pH 7.4

up to 50ml with dH_2O

A 4.2 10% Acrylamide separating gel

30% Acrylamide solution (Flowgen Bioscience Ltd., Nottingham, UK)

1.5 M Tris-base pH 8.8

10% SDS

dH_2O

10% ammonium persulphate

1 % TEMED

A 4.3 4% Acrylamide stacking gel

30% Acrylamide solution

0.5 M Tris- HCL pH 6.8

10% SDS

dH_2O

10% ammonium persulphate

5% TEMED

A 4.4 Sample buffer

0.5M Tris pH 6.8

50% glycerol

10% SDS

0.4% bromophenol blue

dH_2O

A 4.5 Reducing sample buffer

Sample buffer (above)

10% β-mercaptoethanol

A 4.6 Running buffer

0.025M Tris, pH 8.3

0.192M glycine

0.1% SDS

dH_2O

A 4.7 Transfer buffer

0.025M Tris pH 8.3

0.2M Glycine

dH_2O

A 4.8 Washing Buffer (PBS-T)

PBS

0.05% Tween 20

A 4.9 Blocking Solution

5% skimmed milk powder

PBS-T

A 4.10 Coomassie Blue Stain

30% Methanol

10% Acetic acid

2.5g Commassie Brilliant Blue

Make up to 500ml with dH_2O

A 4.11 Commassie Destain

30% Methanol

10% Acetic acid

Make up to 500ml with dH_2O

A 5 Zymography buffers and solutions

A 5.1 12% Gelatin-substrate Acrylamide resolving gel

dH_2O

30% Acrylamide mix

1.5M Tris (pH 8.8)

10% SDS

10% Gelatin

10% Ammonium persulphate

1% TEMED

A 5.2 Stacking gel

dH_2O

30% Acrylamide mix

0.5M Tris (pH 6.8)

10% SDS

10% Ammonium persulfate

5% TEMED

A 5.3 Running buffer

0.25M Tris base

0.52M Glycine

1% SDS

dH_20 up to 1 litre

A 5.4 Renaturing Buffer

25% Triton X-100

dH_2O

A 5.5 Developing buffer

1M Tris (pH 7.5)

5M NaCl

1M $CaCl_2$

2.5% Triton X-100

Adjust to pH 7.5 with 0.1M HCl and make up to 200ml with dH_2O

A 6 Plasminogen Activator Activity Assay

A 6.1 Tris/NaCl buffer

100mM NaCl

50mM Tris pH 7.4

A 6.2 Tris/Tween80 buffer

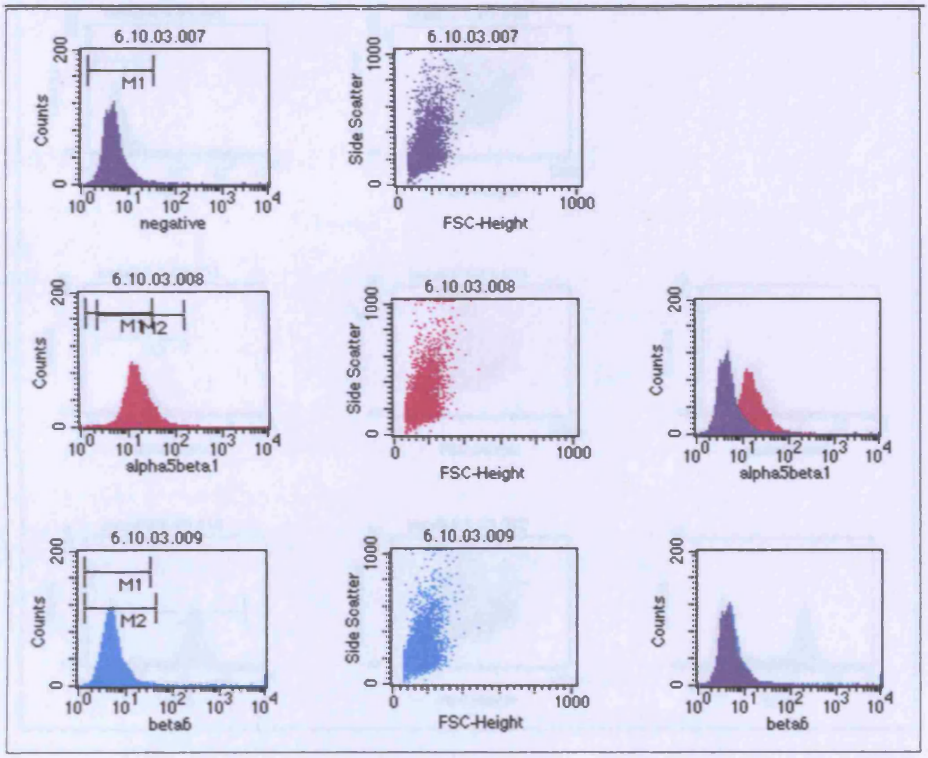
10 ml Tris/NaCl buffer

10% Tween 80

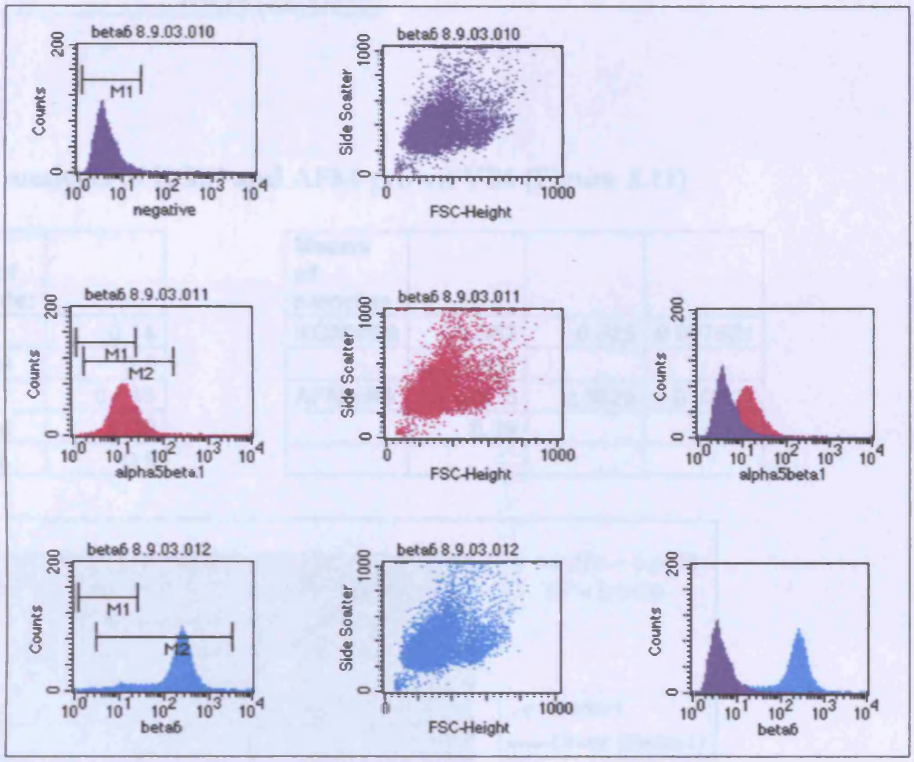
APPENDIX 2

CHAPTER 3

Integrin profile (α5β1 and αvβ6) of C1 cell line.



Integrin profile (α5β1 and αvβ6) of VB6 cell line.



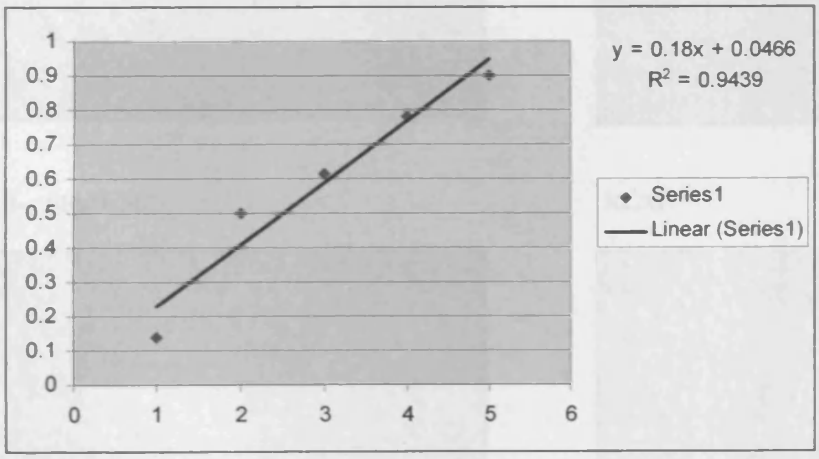
Integrin profile of 3 cell lines (Figure 3.4).

		mean	Stdev
NHK	***α5β1***	4.635	1.746554
	αvβ6	7.355	3.514321
C1		14.94	14.41084
		1.14	0.622254
VB6		2.69	1.088944
		178.78	22.20315

Protein analysis of KGM and AFM-grown VB6 (Figure 3.11)

Means of standards:	
0mg/ml	0.14
2.5mg/ml	0.5
5mg/ml	0.613
7.5mg/ml	0.78
10mg/ml	0.9

Means of samples			
KGMVB6	0.363	*0.425*	*0.087681*
	0.487		
AFMVB6	0.375	*0.3825*	*0.010607*
	0.39		

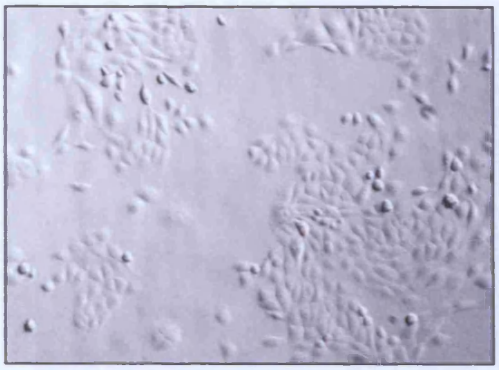


FACS analysis of E-cadherin in KGM and AFM-grown VB6 and C1 (Figure 3.11).

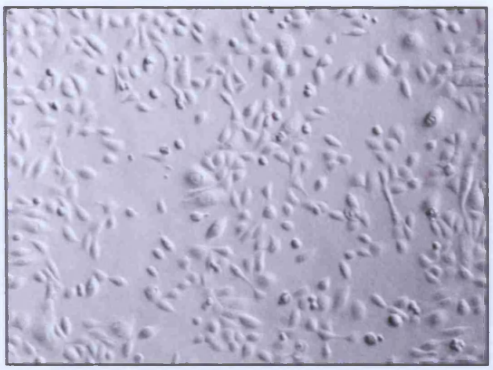
	E-cad	*mean*	*Stdev*
C1-AFM	8.62	9.36	1.046518
	10.1		
VB6-AFM	11.505	11.1125	0.555079
	10.72		

VB6 grown in various medias without serum:

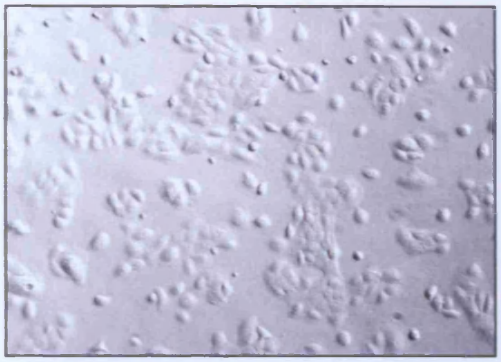
A- SFM+ insulin



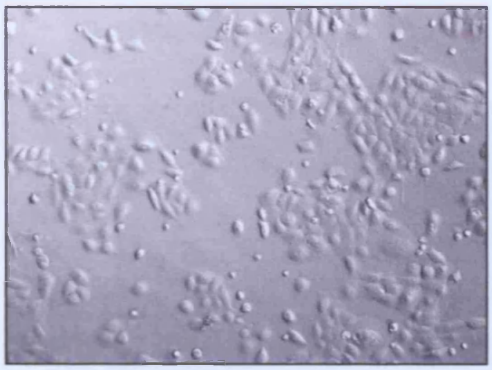
B-SFM+EGF



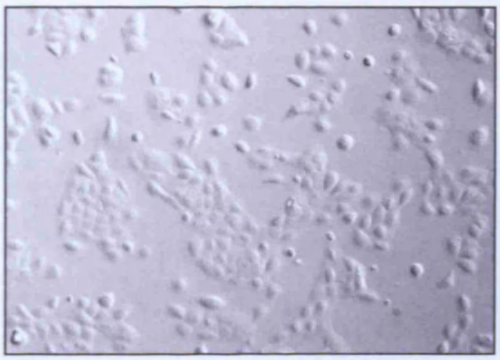
C-SFM+ cholera toxin



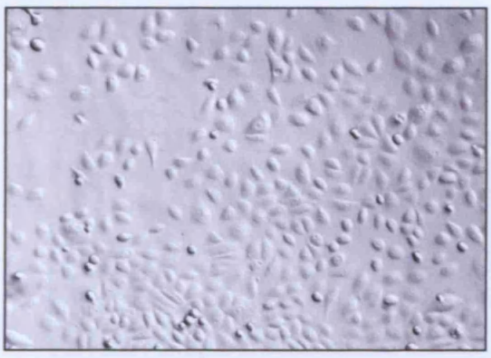
D-SFM+adenine



B- SFM+ HC

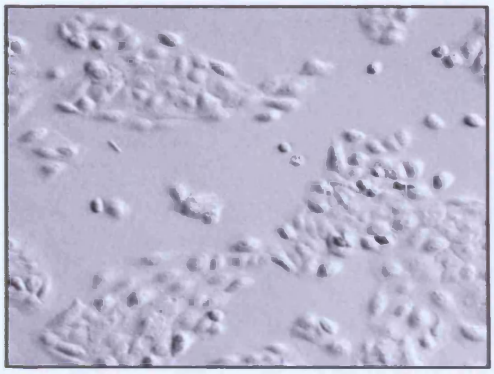


KGM



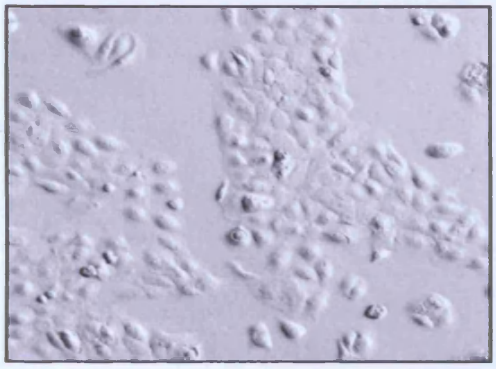
VB6 grown in various medias:

A- AFM+insulin

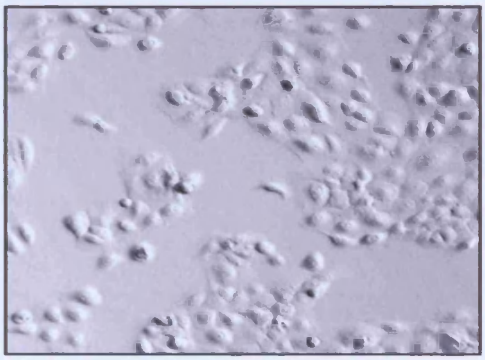


B-AFM+EGF

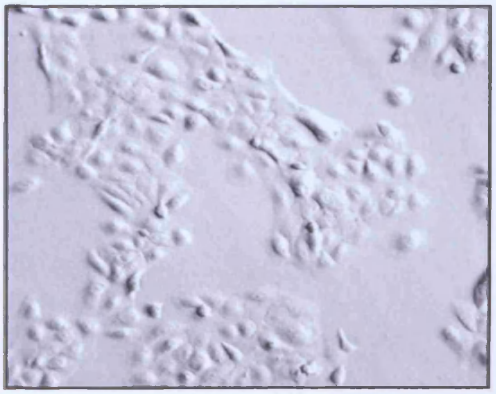
C-AFM+cholera toxin



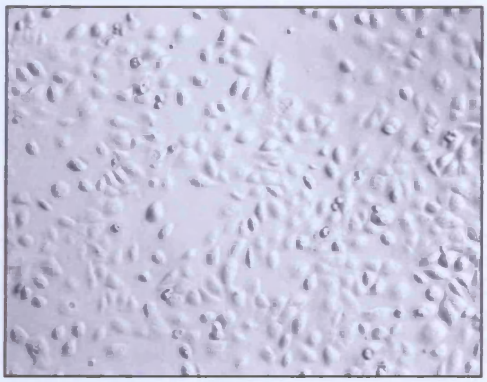
D- AFM+adenine



E-AFM+HC

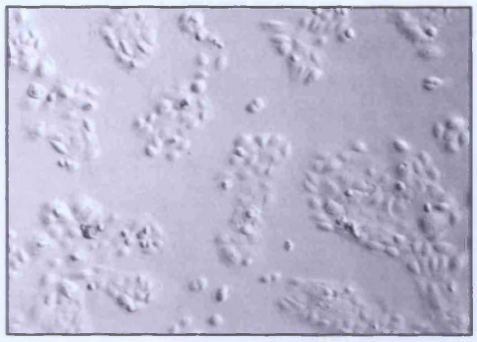


F-KGM

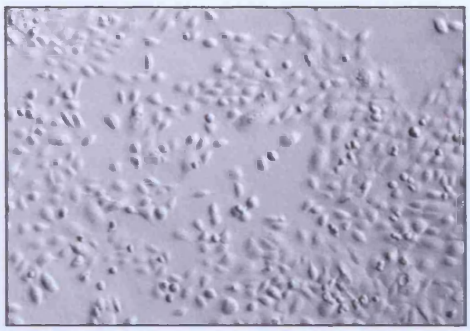


C1 grown in various medias:

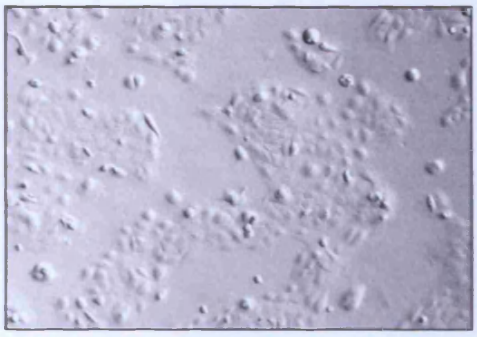
A- AFM+insulin



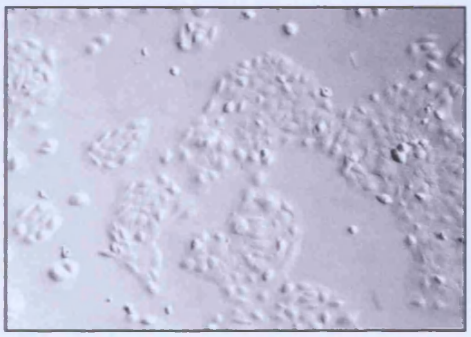
B-AFM+EGF



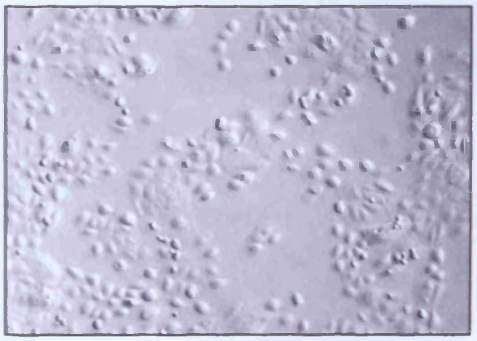
C-AFM+cholera toxin



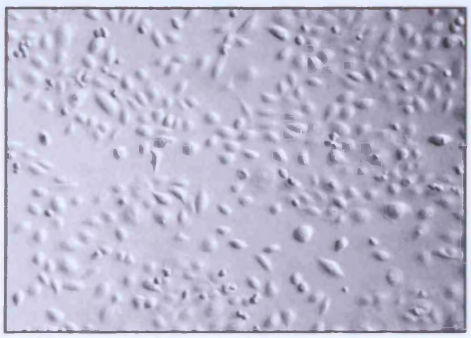
D-AFM+adenine



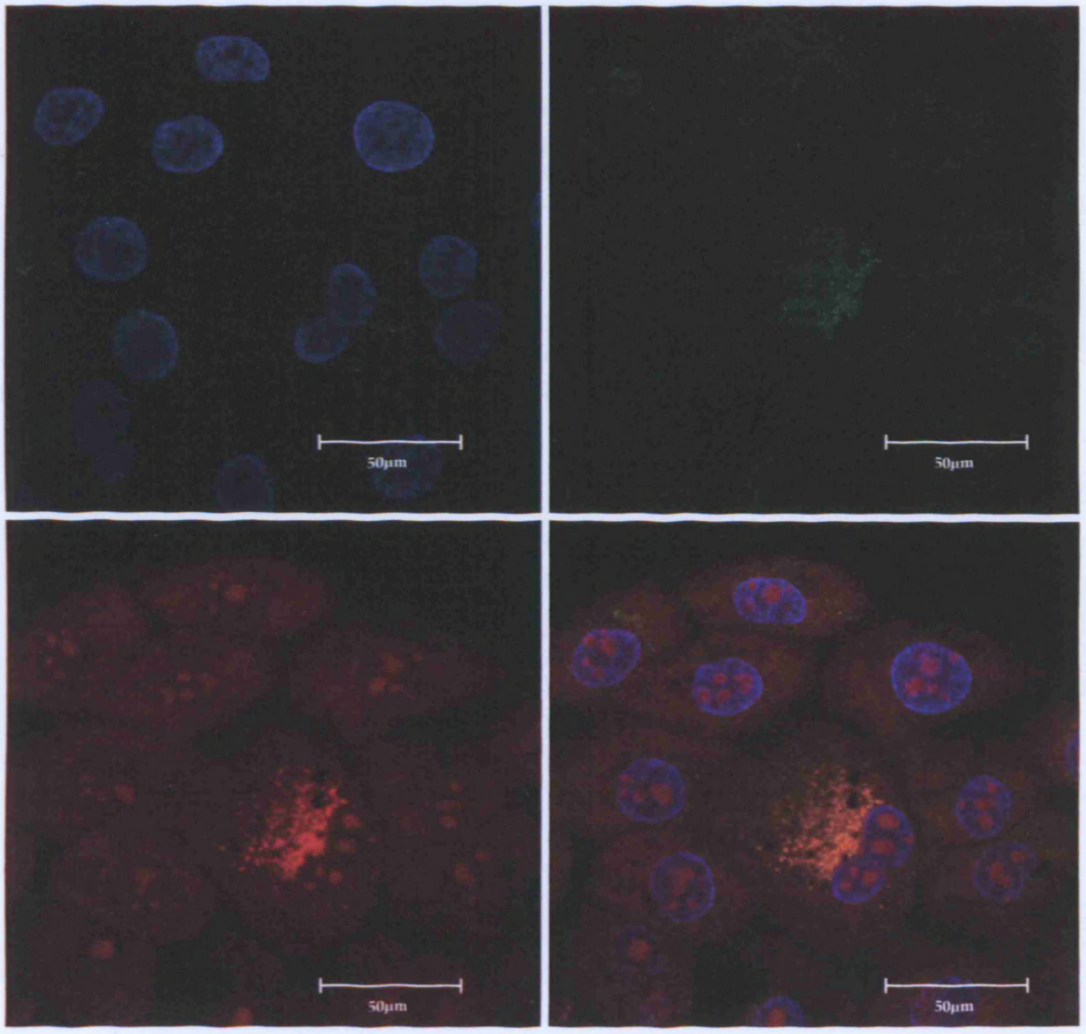
E- AFM+HC



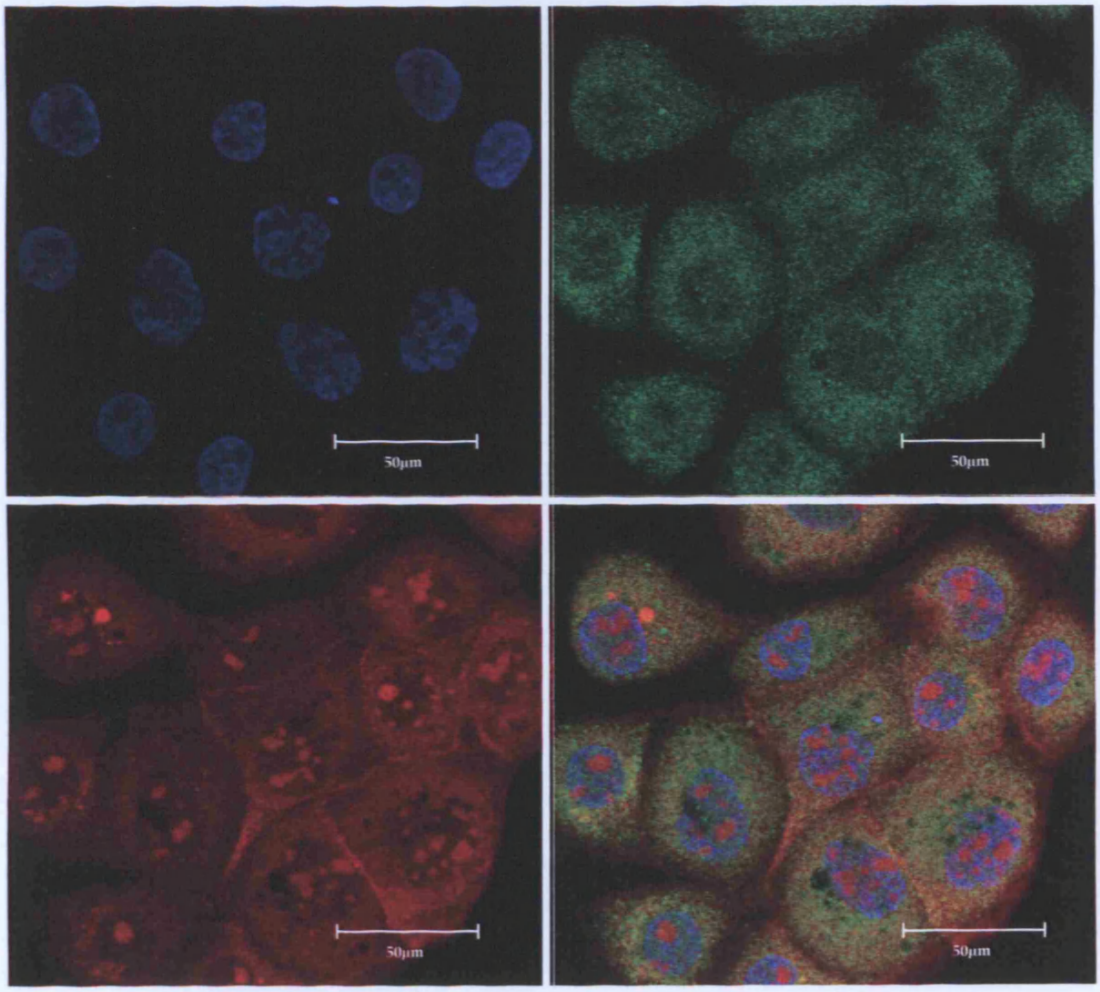
F-KGM



Negative control of Figure 3.12



Negative control of Figure 3.13



CHAPTER 4

Proliferation assay of C1 and VB6 seeded at different densities

C1 T0 30,000			
	ID	mean	st dev
pFN	1.456	1.329	0.110177
	1.272		
	1.259		
120	1.7	1.75	0.050507
	1.801		
	1.749		
control	1.902	1.8885	0.019092
	1.875		
C1 T1 30,000			
	ID	mean	st dev
pFN	1.328	1.467333	0.13706
	1.602		
	1.472		
120	1.175	1.275333	0.097172
	1.282		
	1.369		
control	1.072	1.355333	0.245895
	1.481		
	1.513		
VB6 T0 30,000			
	ID	mean	st dev
pFN	0.862	0.893667	0.10321
	1.009		
	0.81		
120	1.55	1.444	0.094884
	1.367		
	1.415		
control	1.5	2.078333	0.502407
	2.328		
	2.407		
VB6 T1 30,000			
pFN	1.31	1.254667	0.107296
	1.323		
	1.131		
120	1.22	1.106	0.104747
	1.014		
	1.084		
control	1.119	1.114333	0.054151
	1.166		
	1.058		

C1 T0 15,000			
	ID	Mean	st dev
pFN	0.702	0.836333	0.126429
	0.854		
	0.953		
120	0.905	0.925667	0.094707
	1.029		
	0.843		
control	0.759	0.7785	0.027577
	0.798		
C1 T1 15,000			
pFN	0.844	0.829667	0.036665
	0.857		
	0.788		
120	0.938	0.812667	0.12079
	0.803		
	0.697		
control	0.795	0.772667	0.104309
	0.659		
	0.864		
VB6 T0 15,000			
pFN	0.742	0.794	0.093552
	0.902		
	0.738		
120	1.05	0.985	0.101533
	0.868		
	1.037		
control	0.809	1.089	0.404692
	0.905		
	1.553		
VB6 T1 15,000			
pFN	0.611	0.603333	0.110699
	0.489		
	0.71		
120	0.65	0.792	0.149492
	0.948		
	0.778		
control	0.607	0.629	0.057263
	0.694		
	0.586		

Proliferation data of C1 and VB6 on pFN, 120 FN fragment and control

Day1					**Day2**				
C1:	OD	Cell number	Mean	Stdev		OD	Cell number	Mean	Stdev
pFN	0.876	125142.5	118666.3	7058.55	*pFN*	1.071	152999.6	183285.3	26259.69
	0.838	119713.9				1.398	199713.9		
	0.778	111142.5				1.38	197142.5		
120FNfrg	0.913	130428.2	133332.9	14575.86	*120FNfrg*	1.145	163571	161809.1	5707.734
	0.843	120428.2				1.165	166428.2		
	1.044	149142.5				1.088	155428.2		
control	0.894	127713.9	119428.2	7316.462	*control*	1.165	166428.2	163142.5	4510.758
	0.797	113856.7				1.106	157999.6		
	0.817	116713.9				1.155	164999.6		
Day3					**Day4**				
	OD	Cell number	Mean	Stdev		OD	Cell number	Mean	Stdev
pFN	1.145	163571	185428.2	23003.55	*pFN*	1.138	162571	156809.1	13991.49
	1.466	209428.2				0.986	140856.7		
	1.283	183285.3				1.169	166999.6		
120FNfrg	1.886	269428.2	270904.4	15837.4	*120FNfrg*	0.946	135142.5	141428.2	5473.498
	2.012	287428.2				1.016	145142.5		
	1.791	255856.7				1.008	143999.6		
control	1.886	269428.2	252904.4	15230.43	*control*	0.952	135999.6	134952	9614.332
	1.676	239428.2				1.008	143999.6		
	1.749	249856.7				0.874	124856.7		

Day1					**Day2**				
VB6:	OD	Cell number	Mean	Stdev		OD	Cell number	Mean	Stdev
pFN	0.843	168599	169532.3	2722.744	*pFN*	1.757	351399	331332.3	34929.83
	0.837	167399				1.758	351599		
	0.863	172599				1.455	290999		
120FNfrg	0.891	178199	159599	17296.24	*120FNfrg*	1.809	361799	263799	86365.5
	0.72	143999				1.154	230799		
	0.783	156599				0.994	198799		
control	0.72	143999	138265.7	8259.136	*control*	0.918	183599	196799	18074.29
	0.71	141999				1.087	217399		
	0.644	128799				0.947	189399		
Day3					**Day4**				
	OD	Cell number	Mean	Stdev		OD	Cell number	Mean	Stdev
pFN	1.437	287399	297265.7	73101.12	*pFN*	1.868	373599	347665.7	35505.68
	1.148	229599				1.811	362199		
	1.874	374799				1.536	307199		
120FNfrg	1.017	203399	298132.3	100530.7	*120FNfrg*	1.634	326799	372399	40292.93
	2.018	403599				1.936	387199		
	1.437	287399				2.016	403199		
control	1.211	242199	314799	72901.85	*control*	1.598	319599	329599	53110.83
	1.571	314199				1.935	386999		
	1.94	387999				1.411	282199		

Data of migration assay on 120kDa FN fragment (Figure 4.3)

	C1: pFN	C1: 120fr.	C1: control		VB6: pFN	VB6: 120fr.	VB6: control
	Memb.1	Memb.1	Memb.1		Memb.1	Memb.1	Memb.1
	16	23	13		107	153	7
	41	30	8		85	138	2
	45	24	10		82	125	8
	15	21	16		63	136	0
	19	15	5		52	130	3
	22	25	12		74	161	9
	13	14	3		85	159	6
	34	26	11		69	148	9
	20	18	3		74	119	5
Average:	25	21.77778	9		76.77778	141	5.444444
	Memb.2	Memb.2	Memb.2		Memb.2	Memb.2	Memb.2
	9	15	4		88	160	20
	12	15	3		92	161	4
	25	22	12		91	141	4
	32	12	4		76	164	21
	12	9	24		42	152	1
	20	14	10		78	121	5
	10	15	6		91	133	4
	18	23	3		79	136	12
	11	25	10		48	156	2
Average:	16.55556	16.66667	8.444444		76.11111	147.1111	8.111111
	Memb.3	Memb.3	Memb.3		Memb.3	Memb.3	Memb.3
	10	5	12		71	124	18
	13	34	5		65	134	3
	24	8	8		80	121	6
	24	10	10		86	157	18
	9	14	9		96	129	16
	15	13	9		67	152	11
	10	18	9		43	131	14
	14	32	5		53	138	4
	6	18	6		54	136	3
Average:	13.88889	16.88889	8.111111		68.33333	135.7778	10.33333

Migration assay data on 30kDa FN fragment (Figure 4.4)

C1: pFN	Mean	St Dev	VB6: pFN	Mean	St Dev
27	25	2.645751	82	84.33333	4.041452
22			82		
26			89		

C1: 30fr.	Mean	St Dev	VB6: 30fr.	Mean	St Dev
13	13.33333	0.57735	9	8.666667	2.516611
14			6		
13			11		

C1: control	Mean	St Dev	VB6: control	Mean	St Dev
2	4.666667	2.516611	1	3.333333	2.081666
5			5		
7			4		

Migration assay data of VB6 on 120FN fragment with various antibodies (Figure 4.5)

	VB6:120frg	VB6+P1D6	VB6+10D5	VB6+both	VB6+IgG
Memb1	89.77778	43	34	19.33333	69.11111
Memb2	94.22222	82.33333	30.77778	20	67.44444
Memb3	131.3333	66	20.44444	16	53.11111
Mean	105.1111	63.77778	28.40741	18.44444	63.22222
St Dev	22.81756	19.7606	7.081829	2.143033	8.796042

Wound assay data of C1 and VB6 cells on wells coated with pFN, 120 FN fragment and control (Figure 4.6)

T0						Averages	***Mean***	***St dev***
C1 pFN							310.4	103.2376
	387	401	371	385	373	383.4		
	248	219	241	224	255	237.4		
C1 120frg	325	296	326	326	304	315.4	355.2667	37.80441
	404	377	397	390	385	390.6		
	347	368	353	352	379	359.8		
C1 control	382	384	392	391	377	385.2	397.8	10.96722
	399	395	385	412	424	403		
	379	382	406	431	428	405.2		
VB6 pFN	429	439	399	415	416	419.6	415.6	28.21347
	422	406	392	307	401	385.6		
	424	430	438	460	456	441.6		
VB6 120frg	440	418	420	445	415	427.6	412.9333	38.3637
	391	363	344	397	352	369.4		
	440	435	445	450	439	441.8		
VB6 control	383	361	384	379	373	376	397.4667	18.60036
	409	403	409	412	411	408.8		
	447	395	404	390	402	407.6		
T1								
C1 pFN	97	163	147	151	177	147	177.0667	53.46862
	210	263	244	238	239	238.8		
	174	200	121	103	129	145.4		
C1 120frg	190	209	220	219	228	213.2	193.7333	17.52864
	193	164	203	185	199	188.8		
	205	174	175	175	167	179.2		
C1 control	183	201	206	245	247	216.4	298.1333	84.96619
	294	307	281	292	286	292		
	415	399	372	377	367	386		
VB6 pFN							336.9	34.93107
	349	355	370	342	392	361.6		
	264	293	328	327	349	312.2		
VB6 120 frg	335	325	336	315	268	315.8	295.9333	55.53245
	228	212	262	219	245	233.2		
	341	357	320	350	326	338.8		
VB6 control	235	234	255	247	236	241.4	248.2	18.36954
	270	232	208	247	214	234.2		
	286	276	239	258	286	269		

Wound assay data of C1 and VB6 cells incubated with pFN, 120 FN fragment and control (Figure 4.7)

					Mean	St Dev
T0	C1 pFN	324.8667	335.6667	341	333.8444	8.219579
	C1 120frg	296.8667	291.4	334.1333	307.4667	23.2552
	C1 control	312.6667	285.3333	368.8	322.2667	42.55339
	VB6 pFN	394.8667	428.6	420.6667	414.7111	17.63763
	VB6 120frg	342.4667	374.5333	405.0667	374.0222	31.30313
	VB6 control	414.7111	374.0222	380.0778	389.6037	21.95345
T1	C1 pFN	242.2667	255.2667	251.8	249.7778	6.731793
	C1 120frg	200.7333	192.0667	225.9333	206.2444	17.5931
	C1 control	206.8667	194.2	260.6	220.5556	35.25307
	VB6 pFN	280.6	313.4	298.7333	297.5778	16.4305
	VB6 120frg	225.9333	191.4	272.5333	229.9556	40.71594
	VB6 control	281.0667	276.8667	273.0667	277	4.001666
T0-T1	C1 pFN	84.06667	7.475686			
	C1 120frg	101.2222	20.42415			
	C1 control	101.7111	38.90323			
	VB6 pFN	117.1333	17.03407			
	VB6 120frg	144.0667	36.00954			
	VB6 control	112.6037	12.97756			

Wound assay data of VB6 and C1 cell lines on wells coated with pFN, 30kDa FN fragment and control

					Mean	**St Dev**
T0	*C1 pFN*	394.9333	459.0667	333.6	**395.867**	**62.7385**
	C1 30frg	385.9333	381.6667	421.9333	**396.511**	**22.1194**
	C1 control	317.8	422.2	246.4667	**328.822**	**88.3836**
	VB6 pFN	386	337.6	390.2	**371.267**	**29.2317**
	VB6 30frg	362.4	386.7333	299.8	**349.644**	**44.8484**
	VB6 control	311.6667	342.4	330.8667	**328.311**	**15.5252**
T1	*C1 pFN*	258	312.7333	280.8667	**283.867**	**27.4897**
	C1 30frg	236.9333	216.8667	298.7333	**250.844**	**42.6694**
	C1 control	186.3333	288.2667	110.6667	**195.089**	**89.1231**
	VB6 pFN	285.7333	247	300.8	**277.844**	**27.754**
	VB6 30frg	268.6	290.6667	215.9333	**258.4**	**38.3966**
	VB6 control	211.6	262.6667	231.1333	**235.133**	**25.7672**
		T0	T1	**T0-T1**		
T0-T1	*C1 pFN*	395.8667	283.8667	**112**		
	C1 30frg	396.5111	250.8444	**145.667**		
	C1 control	328.8222	195.0889	**133.733**		
	VB6 pFN	371.2667	277.8444	**93.4222**		
	VB6 30frg	349.6444	258.4	**91.2444**		
	VB6 control	328.3111	235.1333	**93.1778**		

Wound assay data of VB6 and C1 cell lines incubated with pFN, 30kDa FN fragment and control

		Mean	St Dev	
T0	C1-pFN	216.9778	18.16619	14.31492
	C1-30	364.7111	7.313903	71.04762
	C1 control	290.3556	13.09736	27.30234
	VB6-pFN	358.8	21.38161	12.69406
	VB6-30	332.6	22.65774	8.366069
	VB6 control	343.9333	23.14054	20.13569
T1	C1-pFN	89.77778	14.31492	
	C1-30	183.6	71.04762	
	C1 control	116.7333	27.30234	
	VB6-pFN	233.9111	12.69406	
	VB6-30	194.4	8.366069	
	VB6 control	182.6444	20.13569	
T0-T1	C1-pFN	127.2	16.24056	
	C1-120	132.7333	19.84057	
	C1-30	181.1111	39.18076	
	control	173.6222	20.19985	
	VB6-pFN	124.8889	17.03784	
	VB6-120	136.5111	8.417903	
	VB6-30	138.2	15.51191	
	control	161.2889	21.63811	

Adhesion assay data for C1 and VB6 cells on wells coated with pFN, 120 FN fragment and control (Figure 4.8)

C1: pFN	Mean	St Dev	VB6: pFN	Mean	St Dev
0.519	0.538	0.016523	0.536	0.540667	0.004509
0.546			0.541		
0.549			0.545		
C1: 120frag.	Mean	St Dev	VB6: 120frag.	Mean	St Dev
0.572	0.561333333	0.018475	0.555	0.519333	0.030892
0.54			0.501		
0.572			0.502		
C1: control	Mean	St Dev	VB6: control	Mean	St Dev
0.459	0.456666667	0.004041	0.408	0.401	0.013
0.452			0.386		
0.459			0.409		

Migration assay data of NHK on pFN and 120kDa FN fragment (Figure 4.9)

	memb1	memb2	memb3		memb1	memb2	memb3
pFN	72	62	60	120frg	47	52	45
	69	77	61		44	56	43
	72	64	59		43	43	39
Average	71	67.66667	60	Average	44.66667	50.33333	42.33333
Mean	66.22222				Mean	45.77778	
St Dev	5.640462				St Dev	4.114113	

Proliferation data of NHK grown on pFN, 120 FN fragment and control (Figure 4.10)

	Mean	St Dev				
pFN			120FNfrg		control	
Day1	46382.83	2231.778	48366.17	660.1767	51599.5	3127.699
Day2	54416.17	5140.606	60366.17	5913.191	48332.83	3493.327
Day3	69816.17	11081.67	71499.5	5935.487	64016.17	14900.7
Day4	64999.5	7210.583	69732.83	7681.363	75832.83	9978.769

CHAPTER 5

Plasminogen activator cell density data

C1:				VB6:			
250,000	ng/ml	Mean	St Dev	250,000	ng/ml	Mean	St Dev
0.132	2.4377	2.359425	0.078275	0.146	3.53355	3.794467	0.966097
0.13	2.28115			0.163	4.864225		
0.131	2.359425			0.139	2.985625		
300,000	ng/ml	Mean	St Dev	300,000	ng/ml	Mean	St Dev
0.133	2.515975	2.28115	0.207096	0.141	3.142175	3.298725	0.341193
0.128	2.1246			0.148	3.6901		
0.129	2.202875			0.14	3.0639		
350,000	ng/ml	Mean	St Dev	350,000	ng/ml	Mean	St Dev
0.141	3.142175	2.90735	0.406729	0.161	4.707675	4.342392	0.352962
0.141	3.142175			0.156	4.3163		
0.132	2.4377			0.152	4.0032		
C1 250,000	2.359425	0.078275					
C1 300,000	2.28115	0.207096					
C1 350,000	2.90735	0.406729					
VB6 250,000	3.794467	0.966097					
VB6 300,000	3.298725	0.341193					
VB6 350,000	4.342392	0.352962					

Plasminogen activator assay data of C1 and VB6 seeded on wells coated with pFN, 120kDa FN fragment and control (Figure 5.1)

COATING VB6							
pFN	ng/ml	Mean	St Dev	120fr	ng/ml	Mean	St Dev
0.857	25.01185	21.92401	2.695743	0.854	24.90968	22.5711	2.350015
0.711	20.03953			0.716	20.20981		
0.731	20.72067			0.786	22.5938		
				plastic	ng/ml	Mean	St Dev
				0.667	18.54102	17.13333	4.756573
				0.74	21.02718		
				0.47	11.83179		

COATING C1							
pFN	ng/ml	Mean	St Dev	120fr	ng/ml	Mean	St Dev
0.208	2.908856	3.385654	0.446653	0.197	2.534229	3.011027	0.825838
0.224	3.453768			0.239	3.964623		
0.234	3.794338			0.197	2.534229		
				plastic	ng/ml	Mean	St Dev
				0.176	1.819032	2.738571	0.810957
				0.221	3.351597		
				0.212	3.045084		

Plasminogen activator assay data of C1 and VB6 incubated with pFN, 120kDa FN fragment and control (Figure 5.2)

INCUBATING VB6							
pFN	ng/ml	Mean	St Dev	120fr	ng/ml	Mean	St Dev
0.579	15.544	16.71329	2.668716	0.688	19.25622	19.19945	6.453989
0.558	14.82881			0.875	25.62488		
0.703	19.76707			0.496	12.71727		
				plastic	ng/ml	Mean	St Dev
				0.749	21.33369	18.24586	2.675011
				0.615	16.77006		
				0.611	16.63383		

INCUBATING C1							
pFN	ng/ml	Mean	St Dev	120fr	ng/ml	Mean	St Dev
0.189	2.261773	3.499177	1.140952	0.265	4.850105	5.746939	1.494762
0.255	4.509535			0.267	4.918219		
0.232	3.726224			0.342	7.472494		
				plastic	ng/ml	Mean	St Dev
				0.187	2.193659	3.624053	1.742907
				0.214	3.113198		
				0.286	5.565302		

Plasminogena activator assay data of C1 and VB6 seeded on wells coated with pFN, 30kDa FN fragment and control (Figure 5.3)

C1 COATING				VB6 : COATING			
PFn	ng/ml	Mean	St Dev	PFn	ng/ml	Mean	St Dev
0.134	4.6537	4.2429	0.4108	0.167	8.0428	8.590533	0.699064
0.126	3.8321			0.17	8.3509		
0.13	4.2429			0.18	9.3779		
30 frag.	ng/ml	Mean	St Dev	30 frag.	ng/ml	Mean	St Dev
0.132	4.4483	4.2429	0.177882	0.157	7.0158	7.974333	1.660228
0.129	4.1402			0.185	9.8914		
0.129	4.1402			0.157	7.0158		
control	ng/ml	Mean	St Dev	control	ng/ml	Mean	St Dev
0.143	5.578	4.722167	0.74295	0.158	7.1185	7.392367	0.388816
0.13	4.2429			0.165	7.8374		
0.131	4.3456			0.159	7.2212		

		Mean	St Dev
C1	pFN	4.2429	0.4108
	30kDa FN frg	4.2429	0.177882
	control	4.722167	0.74295
VB6	pFN	8.590533	0.699064
	30kDa FN frg	7.974333	1.660228
	control	7.392367	0.388816

Plasminogen activator assay data of C1 and VB6 incubated with pFN, 30kDa FN fragment and control (Figure 5.4)

C1: ADDING				VB6: ADDING			
PFn	ng/ml	Mean	St Dev	PFn	ng/ml	Mean	St Dev
0.134	4.6537	4.790633	1.749923	0.146	5.8861	7.152733	1.468047
0.119	3.1132			0.155	6.8104		
0.153	6.605			0.174	8.7617		
30 frag.	ng/ml	Mean	St Dev	30 frag.	ng/ml	Mean	St Dev
0.129	4.1402	3.797867	0.313754	0.162	7.5293	6.673467	0.757013
0.123	3.524			0.148	6.0915		
0.125	3.7294			0.151	6.3996		
control	ng/ml	Mean	St Dev	control	ng/ml	Mean	St Dev
0.145	5.7834	4.893333	0.797717	0.166	7.9401	8.111267	1.803353
0.13	4.2429			0.186	9.9941		
0.134	4.6537			0.151	6.3996		

		Mean	St Dev
C1	pFN	4.790633	1.749923
	30kDa FN frg	3.797867	0.313754
	control	4.893333	0.797717
VB6	pFN	7.152733	1.468047
	30kDa FN frg	6.673467	0.757013
	control	8.111267	1.803353

Plasminogen activator assay data of C1 and VB6 seeded in wells coated with pFN, PHSRN peptide and control.

C1 incubating				**VB6 incubating**			
pFn	ng/ml	Mean	St Dev	**pFn**	ng/ml	Mean	St Dev
0.222	3.199	3.76742	1.48821	0.315	6.23712	6.4462	0.55082
0.212	2.87232			0.323	6.49846		
0.201	2.51297			0.342	7.11916		
0.247	4.0157			0.297	5.6491		
0.315	6.23712			0.33	6.72714		
PHRSN.	ng/ml	Mean	St Dev	**PHSRN**	ng/ml	Mean	St Dev
0.365	7.87052	6.39393	1.86771	0.469	11.268	11.7711	0.40776
0.281	5.12641			0.473	11.3987		
0.284	5.22441			0.495	12.1174		
0.397	8.9159			0.49	11.954		
0.272	4.8324			0.495	12.1174		
control	ng/ml	Mean	St Dev	control	ng/ml	Mean	St Dev
0.235	3.62368	3.92423	0.5325	0.469	11.268	11.9606	1.86314
0.239	3.75435			0.434	10.1246		
0.228	3.395			0.562	14.3061		
0.27	4.76706			0.447	10.5493		
0.249	4.08103			0.539	13.5548		

C1	pFN	3.76742	1.48821
	peptide	6.39393	1.86771
	control	3.92423	0.5325
VB6	pFN	6.4462	0.55082
	peptide	11.7711	0.40776
	control	11.9606	1.86314

Plasminogen activator assay data of C1 and VB6 incubated with pFN, PHSRN peptide and control.

ADDING: C1				ADDING: VB6			
pFN	ng/ml	Mean	St Dev	pFN	ng/ml	Mean	St Dev
0.662	19.81193	18.32279	1.679236	0.688	20.88742	22.07322	3.86189
0.634	18.65371			0.641	18.94327		
0.582	16.50273			0.821	26.38897		
PHSRN	ng/ml	Mean	St Dev	PHSRN	ng/ml	Mean	St Dev
0.669	20.10149	20.915	0.806706	0.718	22.12837	24.72058	2.717001
0.708	21.71472			0.775	24.48618		
0.689	20.92879			0.849	27.54719		
control	ng/ml	Mean	St Dev	control	ng/ml	Mean	St Dev
0.653	19.43965	21.63199	3.410794	0.731	22.66612	23.12113	0.788109
0.664	19.89466			0.764	24.03116		
0.801	25.56167			0.731	22.66612		

ADDING:		
VB6 pFN	22.07322	3.86189
VB6 Frg.	24.72058	2.717001
VB6 contr.	23.12113	0.788109
C1 pFN	18.32279	1.679236
C1 Frg.	20.915	0.806706
C1 contr.	21.63199	3.410794

Zymogram data of supernatants of VB6 and C1 cells exposed to pFN, 120kDa FN fragment and control (Figure 5.6 and 5.8)

	MMP2	Mean	/100000	St dev		MMP9	Mean	/100000	St dev
C1 pFN	98	108.6667	630.2667	13.61372	C1 pFN	853	685.3333	3974.933	148.3723
	104					632			
	124					571			
C1 120	239	258.3333	1291.667	20.59935	C1 120	679	761.3333	3806.667	88.54566
	280					855			
	256					750			
C1 control	159	179.6667	844.4333	32.39341	C1 control	643	639	3003.3	138.0435
	163					499			
	217					775			
VB6 pFN	385	411.3333	2138.933	32.7465	VB6 pFN	1230	1169.667	6082.267	52.30997
	401					1137			
	448					1142			
VB6 120	1125	981.6667	5301	228.6314	VB6 120	1712	1727	9325.8	23.43075
	1102					1754			
	718					1715			
VB6 control	413	373.3333	1978.667	36.22614	VB6 control	1106	1125	5962.5	50.26927
	342					1087			
	365					1182			

Zymogram data of supernatants of NHK cells exposed to pFN, 120kDa FN fragment and control (Figure 5.10)

Gel1								
Lane1	2	3	4	5	6	7	8	9
396	664	656	625	633	635	533	544	412
768	971	1149	1147	1024	1055	1036	1035	963
Gel2								
Lane1	2	3	4	5	6	7	8	9
406	712	622	626	656	675	521	583	454
777	1090	1125	1169	1070	1071	1075	1044	949
MMP-9	pFN	120frg	control		MMP-2	pFN	120frg	control
	664	633	544			971	1024	1035
	712	656	583			1090	1070	1044
	656	635	412			1149	1055	963
	622	675	454			1125	1071	949
	625	533	396			1147	1036	768
	626	521	406			1169	1075	777
Mean	650.8333	608.8333	465.8333			1108.5	1055.167	922.6667
St Dev	34.80469	65.32508	79.16165			72.53068	20.98968	122.3122

Migration assay of C1 and VB6 on 120kDa FN fragment and on the fragment plus TIMP-1 (Figure 6.1)

C1 120	C1 120+TIMP	VB6 120	VB6120+TIMP
memb1	Memb1	memb1	memb1
12	18	44	14
3	18	61	10
7	21	70	23
4	9	63	13
15	3	69	8
4	9	85	14
11	12	67	13
6	8	46	8
7	11	55	8
7.666667	**12.11111**	**62.22222**	**12.33333**
memb2	Memb2	memb2	memb2
6	7	32	18
8	10	12	12
9	9	22	9
7	4	16	13
14	8	27	10
8	4	22	16
4	9	20	19
10	5	18	15
3	7	6	12
7.666667	**7**	**19.44444**	**13.77778**
memb3	Memb3	memb3	memb3
10	8	27	42
10	7	8	25
9	2	29	19
6	4	29	18
8	6	31	6
9	4	13	25
10	3	23	13
6	10	16	7
19	7	23	9
9.666667	**5.666667**	**22.11111**	**18.22222**

Migration assay of C1 and VB6 on 120kDa FN fragment and on the fragment plus Roche inhibitor (Figure 6.2)

C1 120 frg.	C1 120+RI	VB6 120frg	VB6 120+RI
memb 1	memb 1	memb 1	memb 1
5	6	106	116
34	32	140	148
73	33	138	128
53	25	116	102
21	9	104	101
51	31	124	102
21	27	76	81
43	19	119	117
11	18	96	100
34.66667	**22.22222**	**113.2222**	**110.5556**
memb2	memb2	memb2	memb2
11	4	108	28
25	12	131	77
44	15	98	107
20	5	112	52
12	9	92	48
55	10	102	56
24	7	80	21
27	16	91	97
20	3	74	40
26.44444	**9**	**98.66667**	**58.44444**
memb3	memb3	memb3	memb3
10	5	152	22
32	5	157	88
42	37	139	93
41	10	86	46
11	5	75	11
37	10	94	44
10	5	80	29
17	2	151	63
15	5	110	13
23.88889	**9.333333**	**116**	**45.44444**

Zymogram data of VB6 cells plated on pFN, 120 FN fragment and control and in the presence of S203580 inhibitor and U-0126 (Figures 6.10 and 6.11).

Gel1	**SB203580**							
pFN++	pFN++	pFN--	pFN--	120frg++	120frg++	120frg--	120frg--	
565	666	1058	1064	618	484	554	773	pro-MMP-9
173	119	155	166	251	264	249	277	pro-MMP-2
617	803	692	852	672	612	615	626	MMP-2
Gel2	**U-0126**							
pFN++	pFN++	pFN--	pFN--	120frg++	120frg++	120frg--	120frg--	
182	209	748	764	198	225	633	617	pro-MMP-9
18	27	225	194	295	569	321	258	pro-MMP-2
205	193	745	749	669	543	581	619	MMP-2
835	782							
Gel3	**SB203580**			**U-0126**				
contr++	contr++	contr--	contr--	contr++	Contr++	contr--	contr--	
616	535	1051	1044	245	201	1063	890	pro-MMP-9
297	283	279	269	332	276	308	461	pro-MMP-2
1009	859	802	910	673	681	883	2345	MMP-2

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